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PROBES OF THE STRUCTURE AND MECHANISM OF
PHOSPHOENOLPYRUVATE SYNTHETASE

by



SUREE NARINDRASORASAK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PROBES OF THE STRUCTURE AND MECHANISM OF PHOSPHOENOLPYRUVATE SYNTHETASE submitted by SUREE NARINDRASORASAK in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Date March 28, 1977

ABSTRACT

Phosphoenolpyruvate synthetase of Escherichia coli has been shown to be a dimer of molecular weight 150,000. The constituent subunits appear to be identical, as judged by the appearance of only one protein band of molecular weight 77,000 on gel electrophoresis under denaturing conditions and from the results of tryptic fingerprinting. The enzyme tends to dissociate to monomers at low protein concentration, but the tendency is much diminished in the phosphoenzyme form, suggesting that enzyme phosphorylation is accompanied by a structural rearrangement in the subunit contact domain. The enzyme appears to show half-of-the-sites reactivity with respect to its phosphorylation by ATP. Several lines of evidence, including identification of 3-phosphohistidine in alkaline digests of the phosphoenzyme, indicate that a histidyl residue is the site of phosphorylation. The enzyme is inhibited by divalent metal ion such as Ca^{2+} , Cu^{2+} and Fe^{2+} . It is strongly inhibited by oxalate, an analogue of enol-pyruvate. The inhibition is competitive with respect to pyruvate.

Phosphoenolpyruvate synthetase is sensitive to sulfhydryl modification by various reagents, namely N-ethylmaleimide, 5,5'-dithio-(bis)-nitrobenzoic acid and iodoacetamide. Oxalate partially protects the enzyme from these reagents at all pH's studied. Oxalate together with ATP completely protects the enzyme from NEM modification at pH 7.0. However, the protective effect of ATP is pH-dependent, leading to the conclusion that the effect is due to structural change accompanying phosphorylation of the enzyme. The enzyme is also inactivated by bromopyruvate. Pyruvate, but not ATP, protects the enzyme from the reagent, and it has been

found that 2 SH- groups of the enzyme were protected by pyruvate from alkylation by bromopyruvate.

Other chemical modification studies also reveal the presence of essential histidyl and arginyl residues of the enzyme.

The enzyme can use various ATP analogues as substrates and from the structure of those nucleotides we infer that the 6-NH₂ group is important for substrate recognition. The enzymatic synthesis of selected ATP analogues from their respective 5'-monophosphates has been achieved using PEP synthetase.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
araAMP	9- β -D-arabinofuranosyl adenosine-5'-monophosphate
araATP	9- β -D-arabinofuranosyl adenosine-5'-triphosphate
dAMP	deoxyadenosine-5'-monophosphate
α AMP	7- α -D-ribofuranosyl adenosine-5'-monophosphate
2'-MeAMP	2'-O-methyl adenosine-5'-monophosphate
3'-MeAMP	3'-O-methyl adenosine-5'-monophosphate
1-MeAMP	1-methyl adenosine-5'-monophosphate
6-MeAMP	N ⁶ -methyl adenosine-5'-monophosphate
6,6-Me ₂ AMP	N ⁶ ,N ⁶ -dimethyl adenosine-5'-monophosphate
6,2'-Me ₂ AMP	N ⁶ ,2'-O-dimethyl adenosine-5'-monophosphate
CMP	cytidine-5'-monophosphate
CoA	Coenzyme A
DTNB	5,5-dithio-(bis)-nitrobenzoic acid
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediamine tetraacetate
F-1,6-P ₂	fructose-1,6-diphosphate
FMP	formycin-5'-monophosphate
GMP	guanosine-5'-monophosphate
IMP	inosine-5'-monophosphate
MOPS	morpholinopropane sulfonic acid
M.W.	molecular weight
NEM	N-ethylmaleimide

NMP	nucleoside-5'-monophosphate
PEP	phosphoenolpyruvate
PEP syn- thetase	phosphoenolpyruvate synthetase
P _i	inorganic phosphate
PRR	proton relaxation rate
SDS	sodium dodecyl sulfate
TLCK	tosyl-L-lysine chloromethyl ketone
TPCK	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone
Tris	tris-(hydroxymethyl)aminomethane
TuMP	tubercidine-5'-monophosphate

CHAPTER I

INTRODUCTION

The utilization of food materials by living organisms proceeds via specific sequences of enzymic reactions which supply the precursors of cell constituents and the energy necessary for biosynthetic and other endergonic processes. In microorganisms one of the major intermediates that fulfils the requirement for both biosynthesis and energy supply is phosphoenolpyruvate (PEP). For example, the biosynthesis of hexose, the pentose moieties of nucleic acids, aromatic amino acids, histidine and serine each necessitate the continuous supply of PEP. PEP is channelled into tricarboxylic acid cycle intermediates via the PEP carboxylase reaction to form oxalacetate, thus providing precursors for cell constituents. It has been long understood that PEP is generously supplied from the catabolism of carbohydrate by the glycolytic pathway. If the organisms are grown on C_4 -compound precursors such as succinate, PEP is provided via the reaction of PEP carboxykinase which converts oxalacetate to PEP. However, when cells are grown in C_3 compounds other than PEP itself, the formation of PEP from pyruvate must occur. It is known that pyruvate and PEP are interconvertible through the reaction catalyzed by pyruvate kinase but that the equilibrium of that reaction lies far to the side of pyruvate formation:



Although the net formation of PEP from pyruvate through reversal of pyruvate kinase reaction has been achieved (1), this requires either that a very high ratio of ATP:ADP is maintained or that the reaction is

pulled to the left by removing one of the products. Thus, it is considered unlikely to occur to a significant extent under physiological conditions.

An energetically favorable direct synthesis of PEP from pyruvate and ATP was first reported in 1965 by Cooper and Kornberg (2). They isolated mutants of E. coli which, while still able to grow on glucose, glycerol or acetate, were unable to grow on pyruvate or lactate as sole carbon source. The extracts of these mutants contained pyruvate kinase and PEP carboxylase in quantities comparable to those of the wild type, indicating that the step missing from the mutants must involve some other enzyme. The cell-free extract of wild type E. coli grown on lactate catalyzed the removal of pyruvate from solutions when incubated with pyruvate, Mg^{2+} and ATP. This reaction catalyzed by E. coli extracts was distinct from the reaction catalyzed by pyruvate kinase since AMP and P_i rather than ADP were found as the other products of the reaction. The enzyme involved has been called PEP synthetase. It catalyzed the following overall reaction:

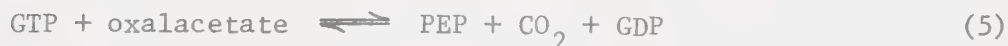


A similar enzyme catalyzed the conversion of pyruvate to PEP has also been reported to operate in some microorganisms and plants (3 - 5). This enzyme, generally referred to as pyruvate, phosphate dikinase, differs from PEP synthetase in that inorganic phosphate replaces water as a substrate, giving the following reaction:



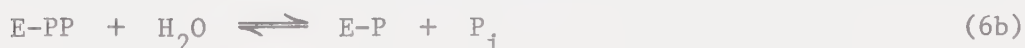
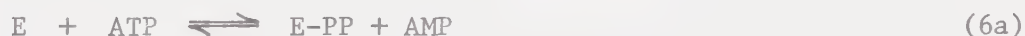
The direct formation of PEP from pyruvate by reaction of PEP synthetase

or pyruvate, phosphate dikinase would be an alternative to the two-step pathway known to occur in animals and yeast that use the combination of pyruvate carboxylase and PEP carboxykinase, which catalyzes reactions (4) and (5) respectively:



However, in both cases the formation of PEP is achieved at the expense of two high energy bonds of ATP.

It was initially proposed (6) that the overall reaction of PEP synthetase involves the transfer of a pyrophosphoryl group from ATP, since γ - ^{32}P -ATP yields exclusively $^{32}\text{P}_i$, whereas β - ^{32}P -ATP gives rise to ^{32}P -PEP. By studying exchange reactions carried out between PEP synthetase and a variety of labelled compounds, Cooper and Kornberg (6) proposed that the reaction involves the following sequence of steps:

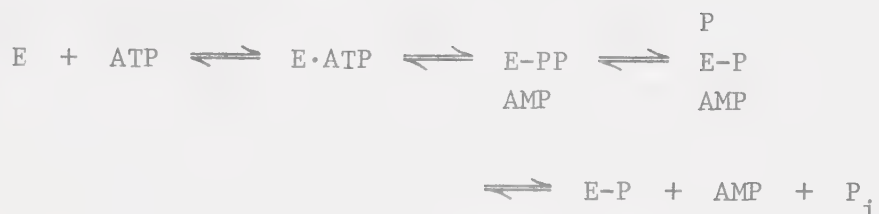


A phosphoenzyme form rather than a pyrophosphoryl enzyme has been isolated following reaction of the enzyme with ATP or PEP (7), but this result provides no evidence about the transient formation of a pyrophosphoryl enzyme since such a form would be expected to be quickly degraded by hydrolysis (step 6b) before isolation. Berman and Cohn (8) have investigated in detail the possibility of the formation of pyrophosphoryl

enzyme intermediate by using ATP analogues, in which the terminal bridge oxygen of ATP is replaced by a $-\text{CH}_2-$ or $-\text{NH}-$ group, which are resistant to hydrolysis at the γ -phosphate of the reaction 6b. They found that though APPCP inhibited the reaction of PEP synthetase competitively with ATP it did not give rise to AMP when it was used as substrate. The formation of the methylene-pyrophosphoryl intermediate by reaction with the analogue was also tested by an investigation of catalyzed exchange between APPCP and ^{14}C -AMP. If APPCP reacts with PEP synthetase to form E=PCP , an exchange of ^{14}C -AMP with APPCP should occur in the absence of P_i , analogous to the AMP-ATP exchange catalyzed by the enzyme (see step 6a). It has been found (8) that only a very slow exchange (less than 1% of that of $\text{AMP} \rightleftharpoons \text{ATP}$) occurs between AMP and APPCP. These results gave no evidence that these compounds could react with PEP synthetase to form an analogue of the postulated pyrophosphoryl enzyme intermediate.

The second approach used by Berman and Cohn (8) was to study the rate of $\text{P}_i - \text{H}_2^{18}\text{O}$ exchange. They predicted that if equations 6a and 6b described a portion of the mechanism of the PEP synthetase reaction, comparable rates of $\text{P}_i \rightleftharpoons \text{H}_2^{18}\text{O}$ exchange are to be expected with EP regardless of its source (i.e. as EP or $\text{E} + \text{ATP}$ or $\text{E} + \text{PEP}$) and the presence of AMP together with ATP should decrease the rate because the concentration of free EP and EPP would be decreased in the presence of AMP. Their experimental findings are completely contradictory to the prediction: the presence of AMP significantly increases the rate of $\text{P}_i \rightleftharpoons \text{H}_2^{18}\text{O}$ exchange. Berman and Cohn (8) have concluded from their studies that there is no free pyrophosphoryl enzyme intermediate involved in the mechanism of PEP synthetase and have proposed a mechanism for the formation of the phosphoryl enzyme intermediate in which the cleavage of

both of the P-O bonds of ATP occurs prior to the dissociation of the products P_i and AMP as follows:



This is similar to the reaction proposed by Andrews and Hatch (9) for the reaction of pyruvate, phosphate dikinase from plants, involving a sequence with only two partial reactions:



However, it is in contrast to the mechanism proposed for pyruvate, phosphate dikinase from Propionibacterium shermanii (5) and from Bacteroides symbiosus (10) for which both pyrophosphoryl and phosphoryl enzyme intermediates have been isolated (11) and the steady state and exchange kinetics have verified the formation of pyrophosphoryl enzyme intermediates (12).

Kinetic studies of PEP synthetase have been done also by Berman and Cohn (13). The kinetic investigations of the forward reaction indicate a ping-pong mechanism with MgATP and pyruvate reacting with different enzyme forms which may be equated with the dephospho- and phosphoenzyme, respectively. From EPR and PRR experiments it was found that a divalent metal ion is required for both partial reactions. The number of binding sites for manganese lies between three and four per mole of enzyme (based upon a molecular weight of 250,000 - ref. 7). Their kinetic experiments indicate that magnesium pyruvate is an active

substrate for the enzyme and that free magnesium and pyruvate combine only with the phosphoenzyme to form kinetically active complexes.

Attempts to study the control characteristics of PEP synthetase have been made by Chulavatnatol and Atkinson (14). They found that the enzyme is strongly inhibited by the products AMP, PEP and also by ADP, and that it responds to variation in the adenylate energy charge (the mole fraction of ATP in the adenylate nucleotide pool plus one-half the mole fraction of ADP) in the manner characteristic of enzymes in biosynthetic pathways. The kinetic competition in vitro between PEP synthetase and the pyruvate dehydrogenase complex from E. coli for the substrate pyruvate was also studied in relation to energy charge (15). It was found that at higher values of charge, conversion to PEP by PEP synthetase (which would lead in vivo to biosynthesis) predominates, and at lower value of charge the partition favors the conversion to acetyl CoA (which would lead to degradation of substrate and regeneration of ATP in vivo).

Despite these detailed investigations, the lack of precise information about the basic structural properties of PEP synthetase such as its molecular weight and subunit composition have prompted us to investigate these aspects of the enzyme. Results of that study are presented in Chapter III.

Most importantly, it is the phosphoenzyme intermediate of PEP synthetase that attracted our attention. Specifically, we wished to identify the nature of the amino acid undergoing phosphorylation. The mechanism suggested for the PEP synthetase reaction stipulates that much of the energy of the high energy bonds of ATP is conserved in the phosphoenzyme intermediate in order to permit synthesis of PEP, whose free energy for hydrolysis is approximately -13 kcal/mole.

A number of enzymes that have reaction mechanisms involving phosphoenzyme intermediate are now known (16 - 30). The phosphoryl group has been found to be linked to proteins through different types of bonds which can be classified into three groups according to their stability at extremes of pH:

(1) Protein-bound, alkali-labile phosphate: characteristic of phosphate esters with the hydroxyl group of serine or threonine. Examples of enzymes that contain this type of linkage are E. coli alkaline phosphatase (16), phosphoglucomutase (17) and the enzymes or proteins which are phosphorylated by the action of protein kinases (18).

(2) Protein-bound, acid-labile phosphate: characteristic of phosphoramidate linkage to N-1 or N-3 of histidine and to ϵ -NH₂ of lysine. Boyer and colleagues (19), who studied the labelling of mitochondrial fractions with $^{32}\text{P}_i$ during oxidation phosphorylation, first observed the presence of phosphohistidine in a biological system. Since then many enzymes have been reported to contain phosphohistidine in their phosphoenzyme forms, namely succinyl CoA synthetase (20), ATP-citrate lyase (21), phosphoglyceromutase (22), nucleotide diphosphokinase (23), lactose specific (lac III) phosphocarrier protein of the Staphylococcus aureus phototransferase system (24), microsomal glucose-6-phosphatase (25), the PEP-dependent phosphocarrier protein of bacterial system (26), pyruvate, phosphate dikinase (27), and acid phosphatase (28).

(3) Protein-bound, acid- and alkali-labile phosphate: characteristic of an acid anhydride linkage of the phosphate to the ω -carboxyl group of aspartic acid or glutamic acid residues. Enzymes containing this type of bond include acetate kinase (29), $(\text{Ca}^{2+}, \text{Mg}^{2+})$ -ATPase of sarcoplasmic reticulum (30) and $(\text{Na}^+, \text{K}^+)$ -ATPase (31).

Of these three types of phosphoryl-enzyme bonds, phosphoramidates and acid anhydrides may be considered high in chemical energy (32) and thus either of these might meet the requirements of phosphorylated PEP synthetase. Indeed, detailed studies on the characterization of the phosphorylated intermediate of PEP synthetase indicate the presence of 3-phosphohistidine in the phosphoenzyme. Results are presented in Chapter IV of this dissertation.

The efficient utilization of pyruvate for growth requires correct partitioning between biosynthesis and catabolism. There must be some control mechanism to ensure that once the PEP has been formed for biosynthetic purposes it should not be used for catabolism; otherwise wasteful cycling between PEP and pyruvate will result. In this regard, a search for allosteric regulation of PEP synthetase by various metabolites has been undertaken. The results, presented in Chapter V, indicate that PEP synthetase is not under physiological control by any of the many metabolites tested.

The use of pseudosubstrates containing chemically reactive groups can provide valuable information about the catalytic centers of enzymes. If the reagent has substrate-like structural features adequate to favor complex formation with the enzyme under consideration, then potential chemical reactivity present in the reagent structure might achieve covalent modification within the active center during the existence of the complex. Halogenomethylketones, inhibitors which retain the main structural features of substrates, as well as possessing pronounced alkylating properties, have given good results with a variety of systems. The usefulness of this approach for enzyme studies has been pioneered with trypsin and chymotrypsin (33, 34) with the development of TLCK and TPCK

which led to the identification of an active center histidine residue. However, when these same reagents were applied to study the active center of papain, the presence of an essential thiol group in this enzyme was shown (35). From these investigations has come the descriptively useful term "active-site - directed reagents" (36). Presently, dozens of active site reagents which irreversibly inactivate selected enzymes have been developed. A similar approach has been applied to PEP synthetase with the use of bromopyruvate, an alkylating agent which is an analogue of pyruvate. These experiments will be described in Chapter VII.

Other approaches to study structure-function relationships of PEP synthetase using various means of chemical modification are also reported.

CHAPTER II

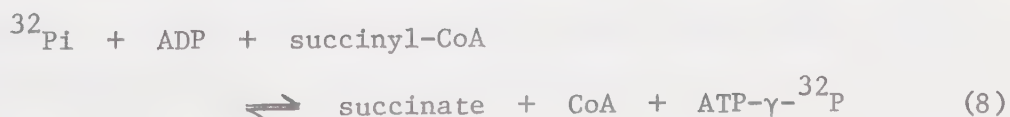
GENERAL MATERIALS AND METHODS

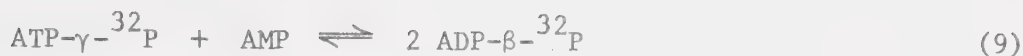
A. MATERIALS

1. Chemicals

Sephadex G-50, G-200 and QAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Tris (ultrapure) and urea (ultrapure) were purchased from Mann Research Laboratories. Ethoxyformic anhydride and phenylglyoxal were from Aldrich Chemical. N-ethylmaleimide, iodoacetamide and 5,5'-dithio(bis)-nitrobenzoic acid were obtained from Sigma Chemical Co. Disc gel electrophoresis supplies were obtained from Canalco. ^{32}Pi , $[^3\text{H}]$ -borohydride and ^{14}C -N-ethylmaleimide were obtained from New England Nuclear. Nucleosides and nucleotides were purchased from the following suppliers: Calbiochem (rAMP, rCMP, rCTP, rGTP); P.L. Biochemicals (rATP, dATP, l-MeATP, 6-MeAMP; rTu); Pfanstiehl, Germany (araA); Raylo, Canada (dAMP); Sigma (rIMP, rITP). Nucleosides were chemically phosphorylated to their 5'-monophosphates by the method of Yoshikawa et al. (37). Adenosine derivatives methylated at the 2'- or 3'-positions were prepared by the method described by Robins et al. (38). 6,6-Me₂ adenosine was prepared from the 6-chloropurine riboside with anhydrous dimethylamine. AMP^{OX-RED} was prepared by the method of Smrt et al. (39).

β - ^{32}P -ATP was prepared from ^{32}Pi by three enzymatic reactions, using succinyl-CoA synthetase, myokinase and pyruvate kinase sequentially as follows:





ATP- γ - ^{32}P was first synthesized by the $\text{Pi} \rightleftharpoons \text{ATP}$ exchange reaction of succinyl CoA synthetase according to Rameley et al. (40). The reaction mixture (2 ml) contained 0.1 μmol of neutralized ^{32}Pi , 0.1 μmol CoA, 10 μmol ATP, 10 μmol MgCl_2 , 50 mM Tris-HCl pH 7.2 and 5 units of purified phosphate-free succinyl CoA synthetase. After incubation at 25° for 20 min the reaction mixture was heated at 100° for 2 min. After cooling, 10 μmol of AMP and 5 units of myokinase were added and the reaction was stopped after 20 min. Then 20 μmol of PEP, 50 μmol of KCl and 5 units of pyruvate kinase were added and the reaction mixture was incubated for a further 20 min. After the pyruvate kinase reaction, the $\text{Pi} \rightleftharpoons \text{ATP}$ exchange catalyzed by succinyl CoA synthetase was repeated in the presence of excess unlabelled Pi in order to eliminate any remaining ATP- γ - ^{32}P . After each step, the radioactive products were monitored by thin layer chromatography on polyethyleneimine cellulose (41). Finally, the reaction mixture was centrifuged and the supernatant was diluted to 100 ml and applied to a column, 2 x 25 cm, of DEAE-cellulose. The product was eluted with linear gradient from 0 - 0.5 M triethylammonium bicarbonate, pH 7.3. The fractions containing the ATP were pooled, concentrated by flash evaporation and the sodium salt of ATP- ^{32}P was precipitated according to the method described by Ramaley et al. (40).

3-Phosphohistidine was synthesized and purified by the method of Hultquist et al. (42), except that diphosphoimidazole (43) served instead of phosphoramidate as the phosphorylating agent.

2. Enzymes

Myokinase, pyruvate kinase, hexokinase and lactate dehydrogenase were obtained from Sigma. Trypsin-TPCK was purchased from Worthington Biochemical Company. Succinyl CoA synthetase was prepared in this laboratory by Mr. E. Brownie from succinate-grown E. coli (Crook's strain) essentially according to the method of Leitzman et al. (44).

3. Cell culture

a. Growth medium

The lactate-containing medium is composed as follows:

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	9.67 g/l
KH_2PO_4	1.91 g/l
NH_4Cl	2.67 g/l
sodium lactate (60%)	7.7 ml/l
yeast extract	0.005 g/l
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.08 g/l
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.027 g/l
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.004 g/l
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.004 g/l

b. Growth of E. coli B

The culture of E. coli B was first grown in 50 ml of tryptone broth overnight at 37°. It was then transferred to a 4 litre of lactate medium for 8 hr. This was inoculated into a 300 l fermentor (Chemap, Switzerland) equipped with automatic temperature and pH controller. Cells were grown overnight at 37°, with stirring at a speed of 50 rpm, and pH was controlled between 7.1 and 7.3 by additions of 1.0 N H_3PO_4 and 1.0 N NaOH. The cells were harvested at late log phase by means of continuous flow centrifugation. The final yields of cells

obtained from 300 l culture were between 1.1 to 1.2 kg, wet weight.

B. METHODS

1. Preparation of PEP synthetase from *E. coli*

PEP synthetase was purified from *E. coli* B grown in lactate medium according to the method of Berman and Cohn (13), with some modifications described in detail in Chapter III.

2. Determination of protein concentration

Protein concentrations during the enzyme purification process were determined by the method of Lowry et al. (45). For other studies which made use of the pure enzyme, the extinction coefficient $E_{1\text{cm}}^{1\%}$ at 279 nm of 8.3 (as determined in Chapter III of this dissertation) was used.

3. Assay of enzyme activity

Enzyme activity in the direction of PEP synthesis was assayed by colorimetric methods and the reverse reaction was assayed by coupling to the lactate dehydrogenase reaction, both as described by Cooper and Kornberg (46).

4. Polyacrylamide gel electrophoresis

Disc gel electrophoresis was carried out in 7% polyacrylamide gels according to the method of Ornstein (47). The procedure of Weber and Osborn (48) was used for gel electrophoresis in the presence of sodium dodecyl sulfate, again using 7% polyacrylamide. The protein was stained with Coomassie blue and the gels were destained by diffusion.

5. Phosphorylation of PEP synthetase

The enzyme was incubated with ATP- β - ^{32}P (or unlabelled ATP) and MgCl_2 in 0.1 M Tris-HCl pH 8.0 for 20 min at room temperature and the

reaction mixture was then passed through a Sephadex G-50 column to separate the small molecular weight compounds from phosphoenzyme.

To determine the stoichiometry of phosphorylation, aliquots of the reaction mixture were removed at time intervals and the [^{32}P]-enzyme was precipitated in 0.2 N perchloric acid in the cold. The precipitate was collected by filtration on a Millipore membrane with 5 μ pore size and was washed several times with cold 0.2 N perchloric acid. The membrane was air-dried and counted for radioactivity in Scintiverse scintillation cocktail. Under these conditions less than 5% of radioactivity was lost from ^{32}P -bound protein.

6. Dephosphorylation of PEP synthetase

In experiments in which dephosphorylated enzyme was to be used, the enzyme was incubated with 5 mM pyruvate and 5 mM MgCl_2 in 0.1 M Tris-HCl, pH 8.0 for 20 min at room temperature and the reagents were separated from the enzyme by means of gel filtration.

7. Amino acid analysis

The enzyme was dialysed against a solution containing 50 mM N-ethylmorpholine-acetic acid pH 8.0, and was lyophilized. The residue was hydrolyzed and its amino acid composition was determined on a Beckman model 120C amino acid analyzer (49).

8. High voltage paper electrophoresis

High voltage electrophoresis at pHs 1.8, 3.5 and 9.0 were performed in a Gilson High Voltage Electrophorator (Model D) equipped with a large fiberglas tank, as described by Dreyer and Bynum (50). The pH 6.5 electrophoresis was performed essentially according to the methods of Michl (51) and Ryle et al. (52). The buffer systems and coolants were similar to those described by Ambler (53). The buffer

system at pH 6.5 is pyridine-acetic acid-water (100:3:900, by vol), pH 1.8 is formic acid-acetic acid-water (1:4:45, by vol), pH 3.5 is pyridine-acetic acid-water (1:10:189) and pH 9.0 is 1% $(\text{NH}_4)_2\text{CO}_3$. Peptides were located with the cadmium-ninhydrin reagent of Heilmann et al. (54) and the Pauley reagent for histidine (55).

CHAPTER III
MOLECULAR WEIGHT, AMINO ACID AND SUBUNIT COMPOSITION
OF PEP SYNTHETASE

A. INTRODUCTION

Since the discovery of PEP synthetase in 1965 (2), some extensive investigations of mechanism (8, 13) and studies of the regulation by energy charge (14) have been reported. Despite these studies, the lack of information about the fundamental molecular structure of the enzyme is surprising. It is imperative that some fundamental physical parameters for the enzyme, namely, molecular weight, number and kinds of subunits, and the ultraviolet extinction coefficient, be established before extensive chemical studies are begun. In this Chapter, we describe experiments to investigate the molecular weight and subunit structure of PEP synthetase from different points of view. The determination of molecular weight and kinds of subunit of proteins by gel electrophoresis in the presence of sodium dodecyl sulfate (48) is a highly reliable method. However, in the case of dissimilar subunits that differ only slightly in molecular weight, this fact may be difficult to detect. A common method of distinguishing between identical and different polypeptide chains in a protein is treatment with the proteolytic enzyme trypsin followed by peptide separation ("fingerprinting"). If the number of lysine and arginine residues in the protein is known, the number of peptides obtained by tryptic digestion is predictable. Thus, a protein that is composed of identical subunits will give rise to fewer different peptides than if the subunits are different.

Molecular weight, subunit structure, amino acid composition and

tryptic mapping of PEP synthetase are reported in this Chapter. A circular dichroism study of the enzyme is also reported which provides some information about protein conformation in terms of content of α -helix, β -structure and random coil.

B. METHODS

1. Purification of PEP synthetase from *E. coli*

PEP synthetase was purified from *E. coli* B grown in lactate-based medium according to the method of Berman and Cohn (13) with some modifications. The preparation of crude extract, the protamine precipitation, the ammonium sulfate fractionation and the DEAE cellulose chromatography were carried out by procedures of Berman and Cohn (13). After the DEAE-cellulose step, re-fractionation with ammonium sulfate and removal of salt, the enzyme was applied to a column of QAE-Sephadex-A50 and eluted with linear gradient from 0.3 M to 0.6 M KCl in 0.03 M potassium phosphate buffer (pH 6.8) containing 2 mM EDTA and 2 mM β -mercaptoethanol. Pooled fractions containing PEP synthetase activity were concentrated by precipitation with ammonium sulfate, 560 g per litre. The precipitate was dissolved in a small volume of 50 mM Tris-HCl (pH 6.8) containing 0.2 mM EDTA and 0.2 mM dithiotreitol, and applied to a column of Sephadex G-200 equilibrated with the same buffer. The pooled enzyme fractions were concentrated by ultrafiltration using Amicon-PM30 membrane. The enzyme was then phosphorylated by treatment with 1 mM ATP and 1 mM MgCl_2 for 30 min at room temperature. The excess of the reagents was removed by passing through a column of Sephadex G-50 equilibrated with 0.3 M KCl in 0.03 M MOPS (pH 6.8) containing 2 mM EDTA and 2 mM β -mercaptoethanol. The protein fractions were then rechromatographed on a column of

QAE-Sephadex-A50 equilibrated with the same buffer used for the previous column and eluted with linear gradient from 0.3 M to 0.6 M KCl in the same buffer (see Appendix I for chromatograms of enzyme from purification steps). All operations were carried out at room temperature. The pure enzyme thus obtained was stored, in dephosphorylated form, in 50 mM Tris-HCl (pH 6.8) containing 0.2 mM EDTA, 0.2 mM DTT and 1 M sucrose at 4°C. It is stable over a period of 12 months under these conditions.

2. Determination of molecular weight of the PEP synthetase subunit

The procedure of Weber and Osborn (48) was used to determine the molecular weight of the PEP synthetase subunit by gel electrophoresis in the presence of sodium dodecyl sulfate, using 7% polyacrylamide. The following proteins were used as standard: phosphorylase (M.W. 94,000 (56)), bovine serum albumin (M.W. 68,000 (57)), glutamate dehydrogenase (M.W. 53,000 (56)), ovalbumin (M.W. 43,000 (58)) and lysozyme (M.W. 14,300 (48)).

3. Molecular weight determinations by sedimentation equilibrium

The enzyme was phosphorylated or dephosphorylated as described in Chapter II, Section B 5, 6. The proteins were then dialyzed against 50 mM Tris-HCl, pH 6.8 containing 0.2 mM EDTA and 0.2 mM DTT, at 20° or at 5° for 24 hr. The molecular weight determinations of these protein samples were performed by conventional low-speed sedimentation equilibrium methods (59) in a Beckman Spinco Model E equipped with an interference optical system. The initial loading concentration of protein for the experiments at 20° was 4 mg/ml and the rotor speed was 4800 rpm; for the experiments at 5°, the protein concentration was 2 mg/ml and the rotor speed was 5200 rpm.

Data were analyzed with the aid of an APL program written for the IBM 360 computer which fits the observed dependence of $\log(\text{concentration})$ versus r^2 to a binomial expression and calculates apparent weight

average molecular weight (M_w) as a function of concentration from the derivative of the binomial at each value of r^2 . The value of $0.73 \text{ cm}^3/\text{g}$ was used for partial specific volume ($\bar{v}$), calculated from the amino acid composition.

4. Amino acid analysis

The amino acid composition studies of PEP synthetase were performed as described in Chapter II, Section B 7. The cysteine and cystine content of protein was determined as cysteic acid by the method of Moore (60). Tryptophan was measured by the method of Karkhanis et al. (61). Corrections for unstable and slowly liberated amino acids were made from results of the kinetics of hydrolysis (49). The amino acid residues were calculated based upon a molecular weight of PEP synthetase of 150,000 daltons.

5. Trypsin digestion and peptide mapping

Approximately 3 mg of PEP synthetase was reduced and carboxymethylated according to the method of Crestfield et al. (62). The carboxymethylated protein, in 0.05 M N-ethylmorpholine - acetic acid (pH 8.0) was digested with 2% (w/w) of TPCK-trypsin at 37° for 8 hr. The peptides were separated by two-dimensional electrophoresis at pH 6.5 and at pH 1.8 as follows: the mixture was lyophilized and dissolved in a small volume of distilled water. The sample was divided into halves, and each was applied to Whatman No. 1 MM filter paper and subjected to electrophoresis at pH 6.5 (acetic acid-pyridine-water) at 3 kv for 30 minutes. The strip of electrophoregram containing peptides was cut out, sewn into a sheet of No. 1 MM paper and resubjected to electrophoresis in a second dimension at pH 1.8, 3 kv for 45 minutes. One of the two air-dried papers was dipped in cadmium-ninhydrin reagent of Heilmann et al.

(54), and the color was allowed to develop in an oven. The other one was dipped in ninhydrin solution which did not contain cadmium, and after drying and the ninhydrin positive spots being marked, it was restained with Pauley reagent (55) for detection of histidine containing peptides.

6. Determination of extinction coefficient

Two independent methods were used. The first involved measurements of the refractive index increment in the analytical ultracentrifuge, essentially as described by Babul and Stellwagen (63). The number of fringes corresponding to each of several loading concentrations of protein were counted with the aid of a microcomparator, and the protein concentration was calculated from the average refractive index increment of 4.10 ± 0.13 fringes $\text{mg}^{-1} \text{ml}^{-1}$ (63), which has been found to be essentially invariant for a wide range of proteins. The extinction coefficient was then estimated from plots of measured absorbance at 279 nm vs. protein concentration.

The second method involved estimation of protein concentration by the microbiuret method (64), using both bovine serum albumin and lysozyme as standards. Concentration of standards was determined from their respective extinction coefficients (65).

7. Circular dichroism

Measurements were made with a Cary model 6001 recording spectropolarimeter at the ambient temperature of the instrument (27°) and at a protein concentration of 1.2 mg/ml. Cells of 0.05 cm and 1 cm path lengths were used. Data are presented as mean residue ellipticity $[\theta]$ ($\text{deg cm}^2 \text{dmol}^{-1}$). The mean residue weight of PEP synthetase was calculated to be 114.

C. RESULTS

1. Specificity and homogeneity of the enzyme

The purification procedure as used by Berman and Cohn (13) for isolation of this enzyme from lactate-grown E. coli B, as slightly modified for the present work, has been found to give good yields and reproducible results. The important innovation introduced here involves phosphorylation of the enzyme to introduce more negative charges, so that rechromatography on QAE-A50 Sephadex eliminates contaminating proteins. After this step, the specific activity obtained in various preparations ranged from 16 to 19 units per mg of protein. Pertinent data for a typical preparation are given in Table I. The preparation obtained consisted of a single component as estimated by ultracentrifugation and polyacrylamide disc gel electrophoresis (Fig. 1).

The enzyme was not found to be extremely cold-labile as reported by Cooper and Kornberg (46). When it was kept in 0.05 M Tris-HCl, pH 6.8 containing 0.2 mM DTT and 0.2 mM EDTA at 4°, without sucrose, its activity decreased by 20% and 33% after a period of 3 days and 1 month respectively. The cold inactivation was reversible by prolonged incubation at 30°.

2. Ultraviolet absorption coefficient of PEP synthetase

PEP synthetase had a maximum absorption at 279 nm with a shoulder at 290 nm. The ratio of the absorbances at 280 and 260 was 1.75. The value for $E_{1\text{cm}}^{1\%}$ at 279 nm as measured by refractometric method in the analytical ultracentrifuge was 8.3, a value confirmed by the microbiuret method as shown in Table II.

3. Molecular weight of the PEP synthetase subunit

When highly purified PEP synthetase which showed a single protein

TABLE I
Purification of PEP Synthetase

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	% Yield	Degree of purifi- cation
Crude extract (1 kg cell paste)	130,000	11,400	0.077	100	1
Protamine sulfate fractionation	11,400	11,300	0.99	99	12.8
Ammonium sulfate fractionation	6,200	9,800	1.59	87	20.6
DEAE-cellulose	4,030	8,360	2.10	74	27.0
QAE-A50 Sephadex	680	7,070	10.4	62	135
Sephadex G-200	318	4,500	14.1	40	183
QAE-A50 Sephadex after phosphorylation	201	3,900	19.7	34	255

Figure 1. Polyacrylamide gel electrophoresis of PEP synthetase.

Left: Analytical run in 7% polyacrylamide in absence of denaturants. Right: Electrophoresis in 7% polyacrylamide in presence of Na-dodecylsulfate.

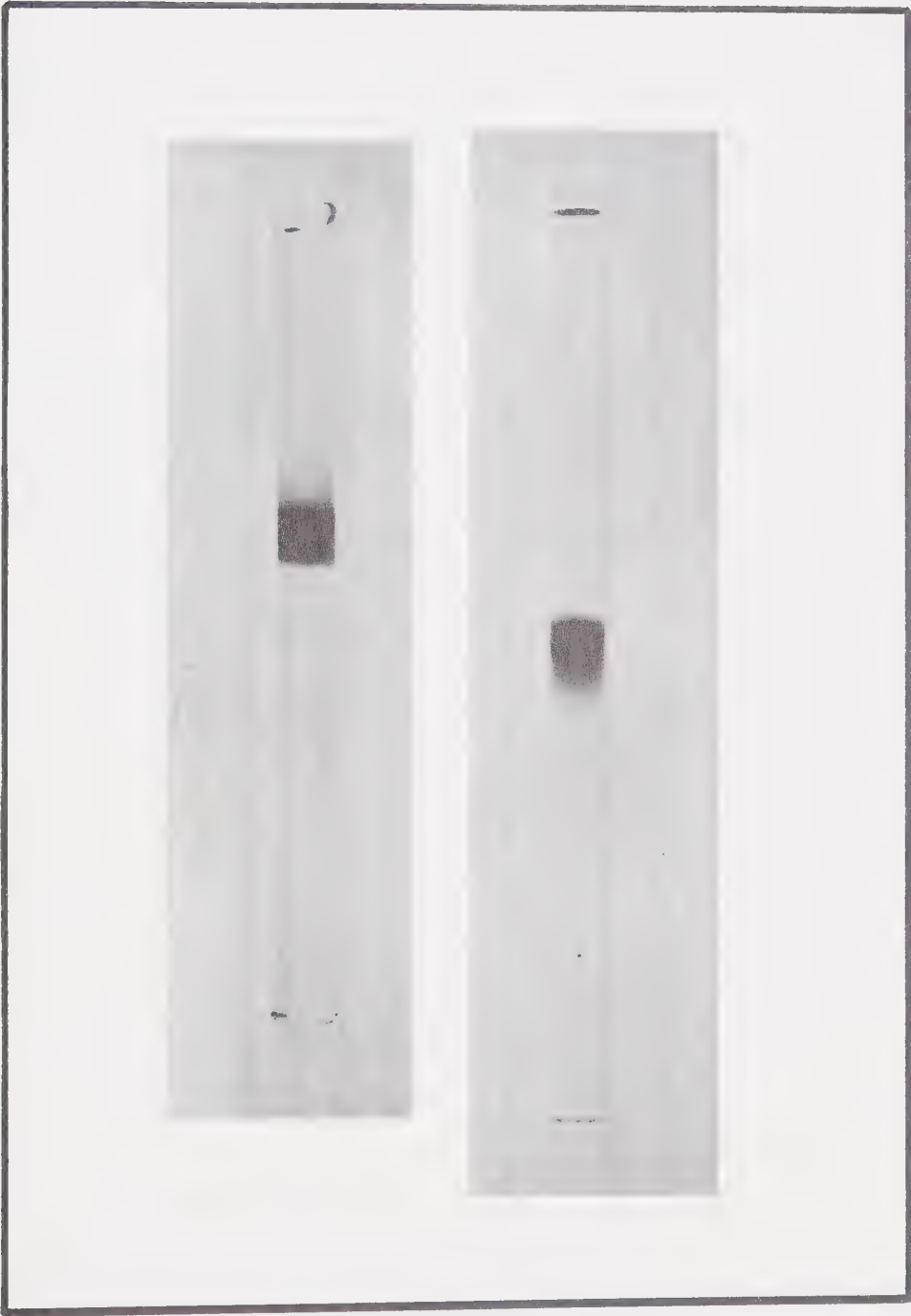


TABLE II

Determination of the Ultraviolet Extinction
Coefficient of PEP Synthetase

Method of protein estimation	Calculated $E_{1\text{cm}}^{1\%}$ at 279 nm
Interference fringe displacement	8.26±0.05
Microbiuret	8.28±0.05

peak in disc polyacrylamide gel electrophoresis was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate, only one band was detected (Fig. 1). The molecular weight of the subunit, determined by comparative mobility to standard proteins, was estimated to be 77,000 daltons (Fig. 2).

4. Sedimentation equilibrium of PEP synthetase

The results of sedimentation equilibrium experiments, representatives of which are given in Fig. 3, show that the molecular weights (M_w) obtained depend both on temperature and enzyme phosphorylation. When studied at 20°, the molecular weight of the enzyme was found to be a function of protein concentration (Fig. 3A). At low concentration the dephosphoenzyme in particular appears to dissociate to a low molecular weight species (ca. 80,000), whereas at high protein concentrations the molecular weight approaches a value of 150,000. When the experiments were performed at 5° (Fig. 3B) both phospho- and dephosphoenzyme were found to exist primarily as dimers, although the dephosphoenzyme still undergoes some dissociation. These results, taken together with the subunit molecular weight of about 77,000, clearly indicate that the PEP synthetase is a dissociable dimer. The strong stabilizing effect of enzyme phosphorylation upon the dimeric structure suggests that phosphorylation is accompanied by a structural rearrangement in the subunit contact domain of the protein.

5. Amino acid composition and peptide mapping

The amino acid composition of PEP synthetase is shown in Table III. The results show a high proportion of acidic residues. The peptide map of the enzyme is shown in Fig. 4, with an estimated total of 58 ninhydrin-positive spots and 11 histidine-containing peptides. In

Figure 2. Estimation of molecular weight of the subunit of PEP synthetase by electrophoresis in 7% polyacrylamide in presence of Na-dodecylsulfate.

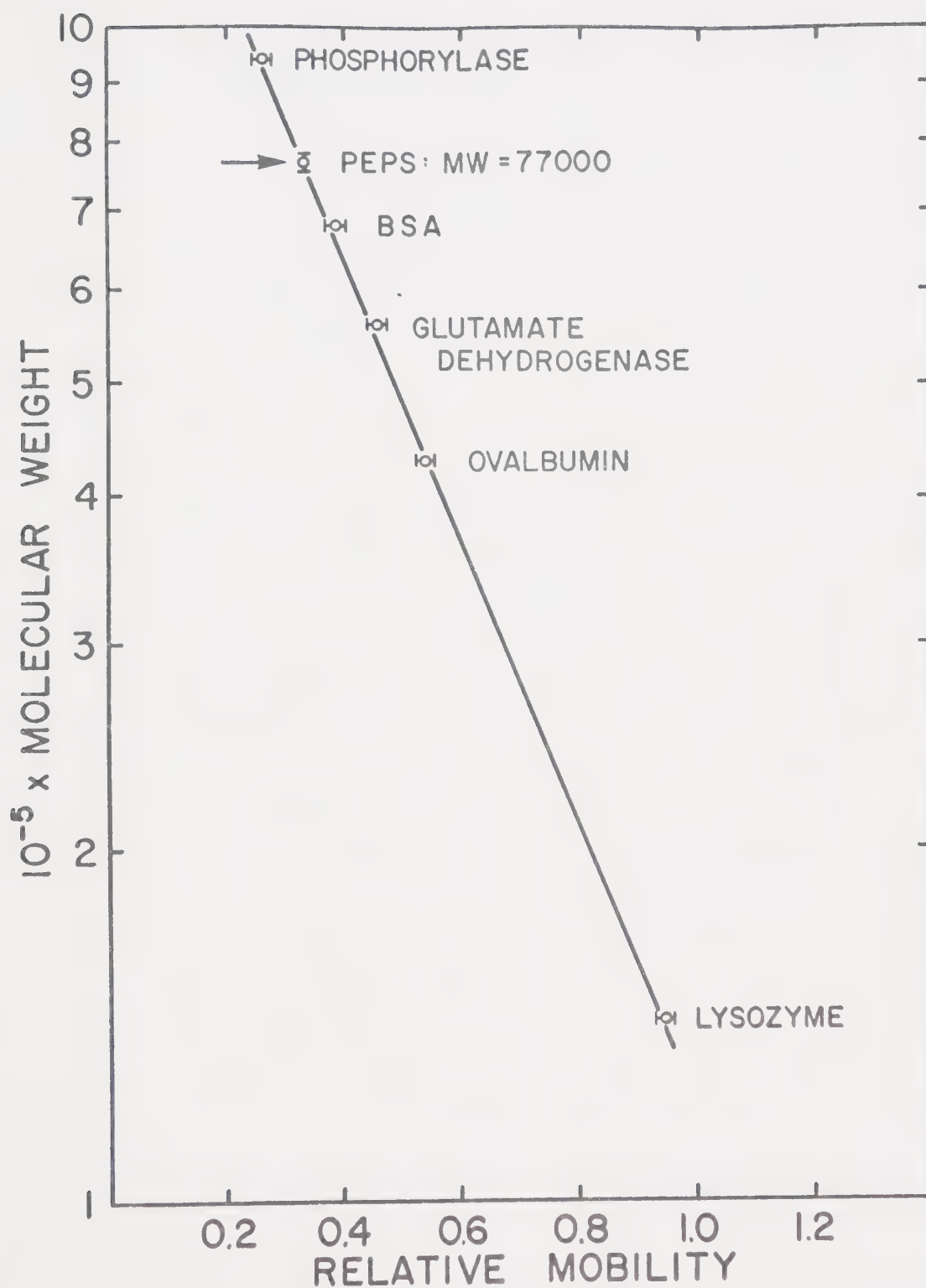
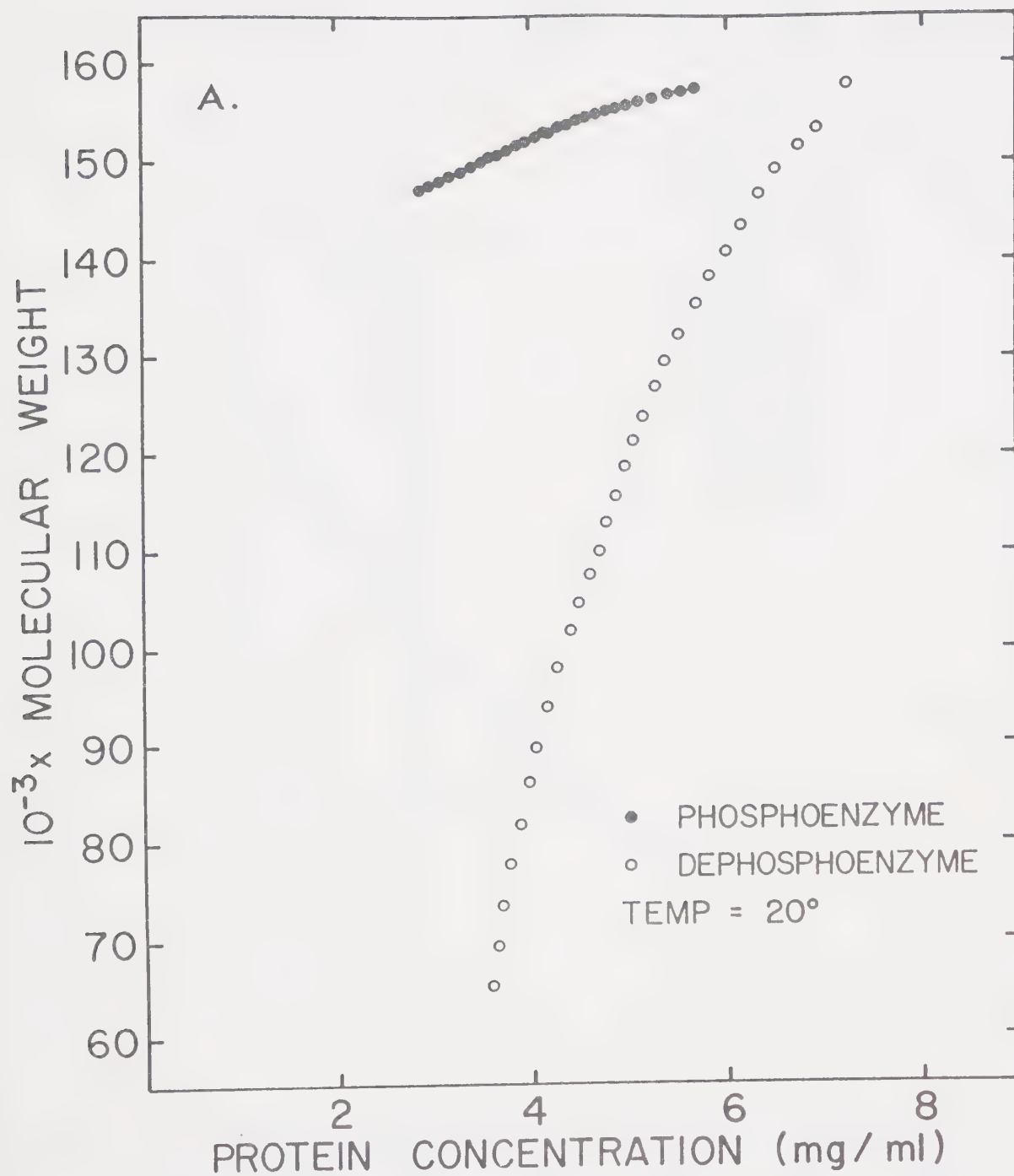


Figure 3. Dependence of weight-average molecular weight (M_w) on the concentration of dephosphoenzyme (O—O) and phosphoenzyme (●—●) forms of PEP synthetase. Experimental conditions were: A, temp = 20°, rotor speed = 4800 rpm, initial loading concentration = 4 mg/ml; B, temp = 5°, rotor speed = 5200 rpm, initial loading concentration = 2 mg/ml.



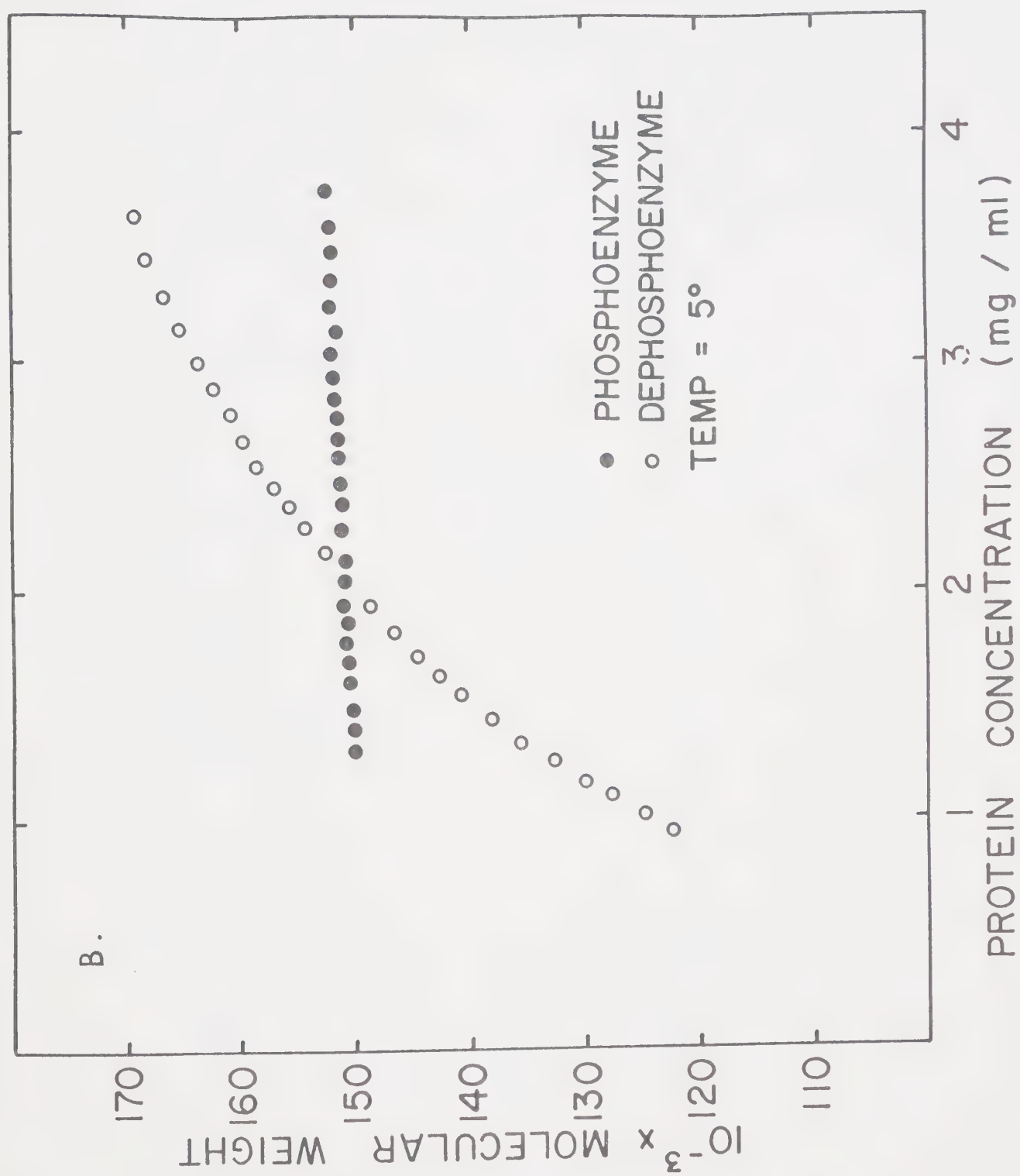
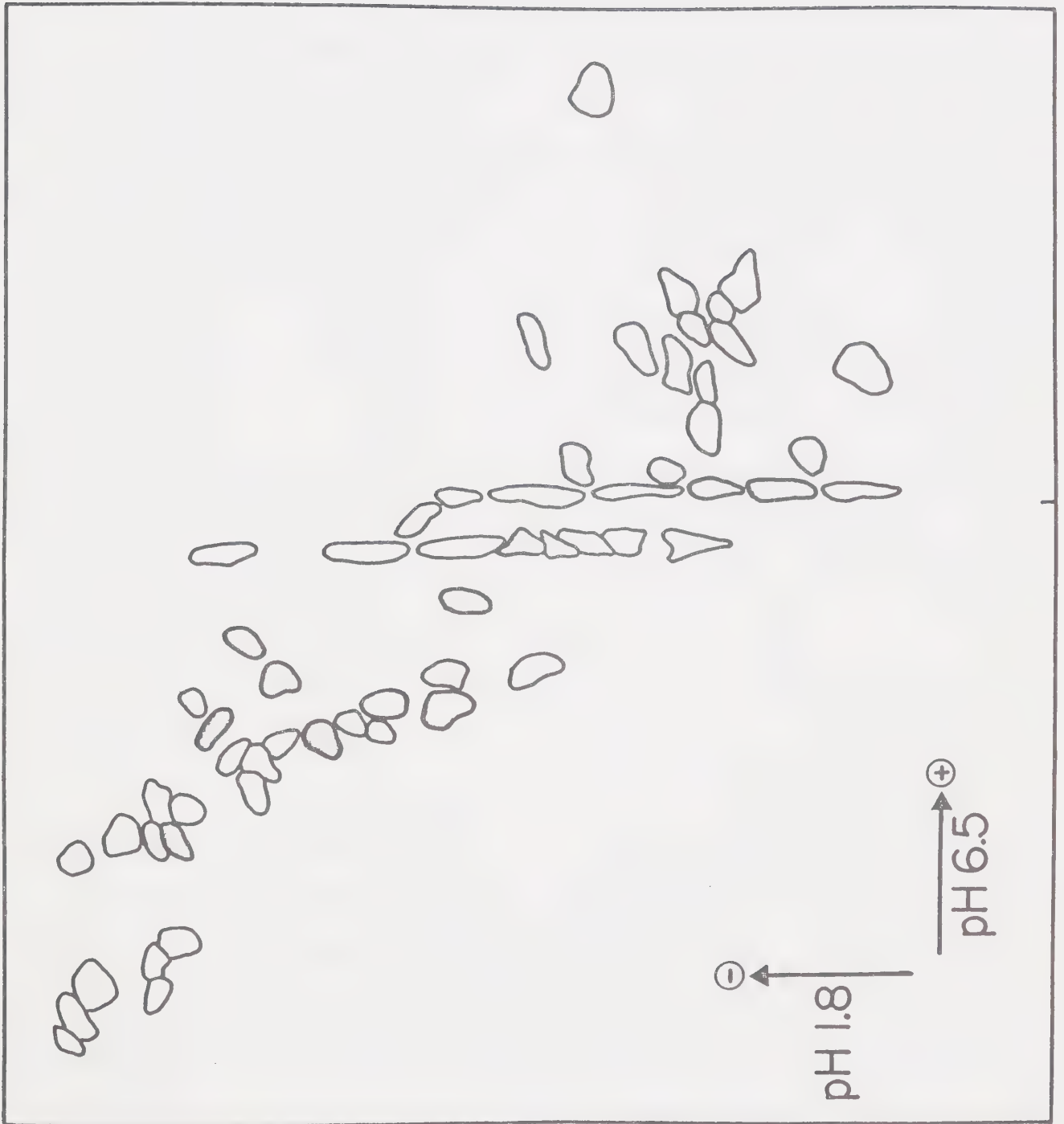


TABLE III
Amino Acid Composition of PEP Synthetase

Amino acid	Residues/ 150,000 daltons
Alanine	118
Arginine	71
Aspartic acid	150
Cysteine	14
Glutamic acid	168
Glycine	110
Histidine	22
Isoleucine	76
Leucine	99
Lysine	52
Methionine	48
Phenyl alanine	56
Proline	52
Serine	74
Threonine	52
Tryptophan	8
Tyrosine	30
Valine	114

Figure 4. Results of two-dimensional high voltage electrophoresis of peptides obtained by digestion of PEP synthetase with trypsin. Shaded spots represent histidine-containing peptides identified with Pauly reagent.



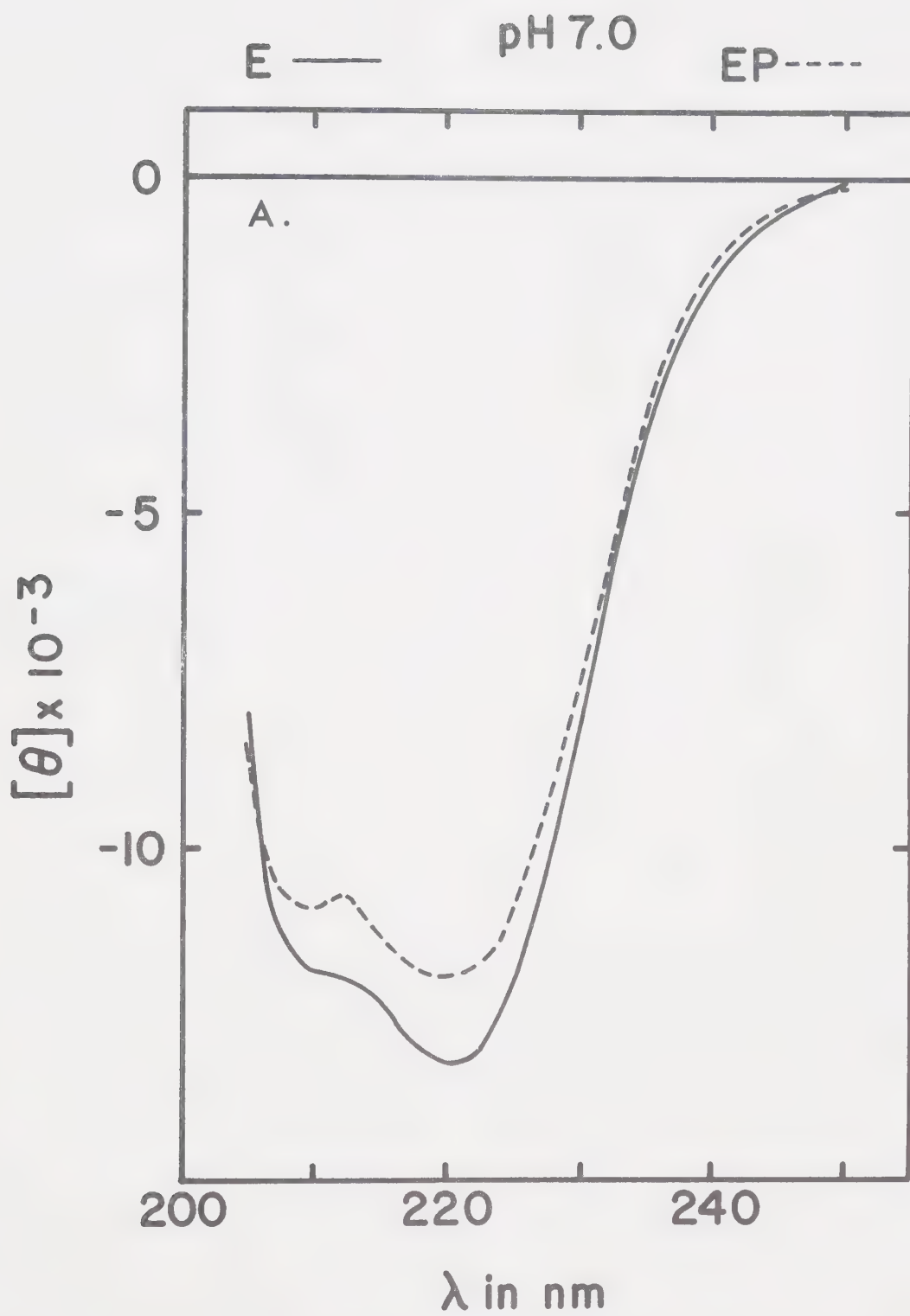
view of the amino acid composition of the enzyme, showing 52 lysine, 71 arginine and 22 histidine residues per molecular of 150,000 molecular weight, the observed number of peptides indicates that the enzyme is a dimer of identical subunits.

6. Circular dichroism

Measurement of the far-ultraviolet circular dichroic spectrum of proteins provides a facile method for the rough quantitation of protein conformation in terms of contributions by α -helix, β -structure and random coil (66). This method was applied to study PEP synthetase in both phosphorylated and dephosphorylated forms, at pH 7.0 (the optimum condition for storage of the enzyme) and at pH 8.0 (the optimum pH for enzyme activity) in order to determine any difference in structure of these two forms of enzyme, especially since there is evidence from the results from sedimentation equilibrium experiments that phosphorylation of the enzyme may be accompanied by some structural rearrangement.

Such spectra for the phosphorylated and dephosphorylated forms of PEP synthetase are given in Fig. 5. Using the method of Chen et al. (67) which relates circular dichroism spectra to the three structural components using reference proteins whose structure has been determined by X-ray crystallography, the calculated fractions of α -helix (f_H), β -structure (f_β) and random coil (f_R) of the enzyme in each experimental condition are presented in Table IV. The results indicate that PEP synthetase contains α -helix, β -structure and random coil in approximately a 1:1:1 ratio. At both pH 7.0 and 8.0, phosphorylation of the enzyme causes minor but significant changes in the proportions of these structures when compared to the dephosphoenzyme. The CD spectrum of the near ultraviolet region (Fig. 6) also seems to indicate that there is

Figure 5. Far-ultraviolet circular dichroism spectra of PEP synthetase at pH 7.0 (A) and at pH 8.0 (B). (—) dephosphoenzyme, (---) phosphoenzyme.



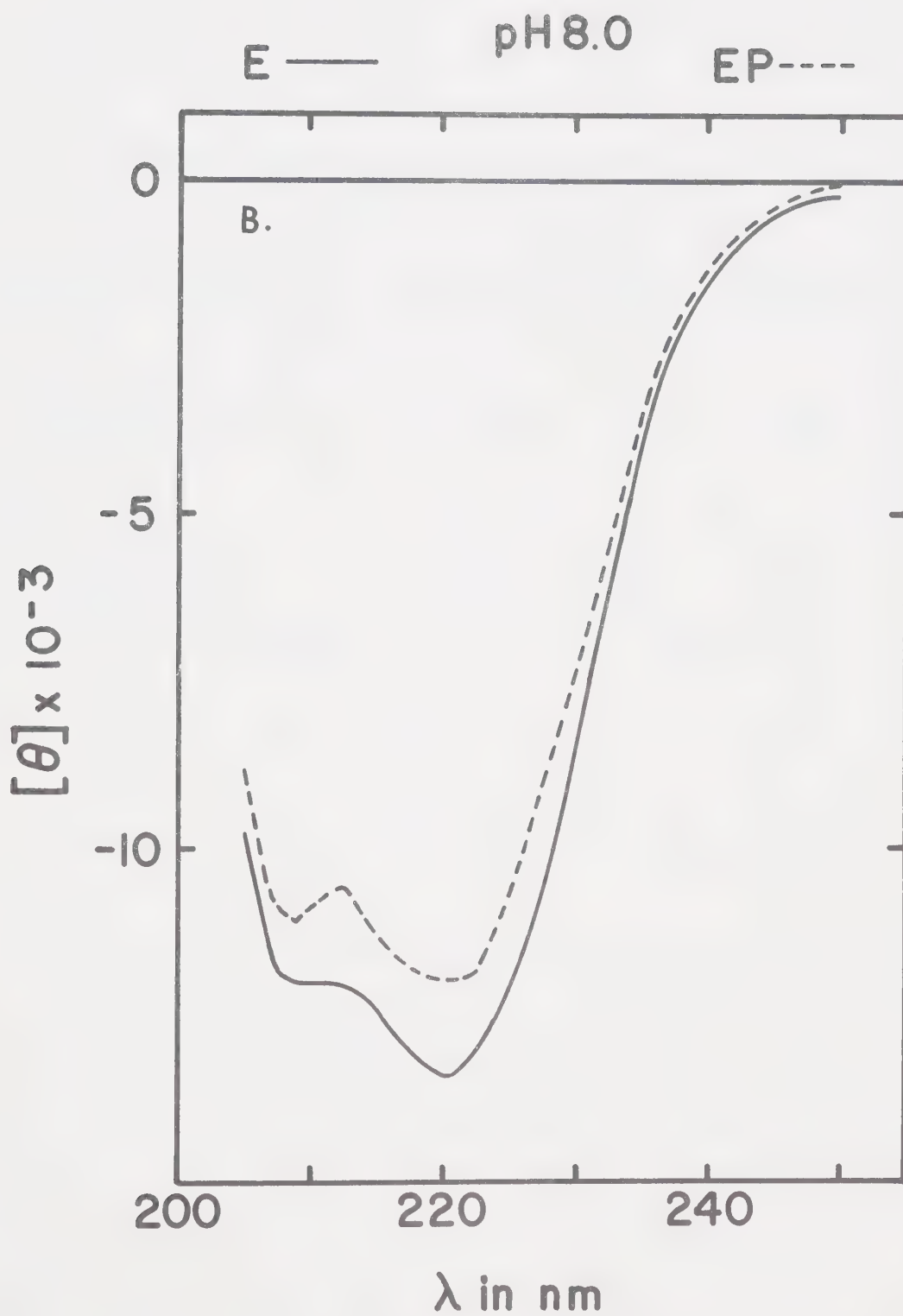


TABLE IV
 α -Helix, β -Structure and Random Coil Content
 of PEP Synthetase

Experimental conditions	α -helix	β -structure	Random coil
pH 7.0			
Dephosphoenzyme	0.368	0.368	0.264
Phosphoenzyme	0.326	0.356	0.318
pH 8.0			
Dephosphoenzyme	0.394	0.284	0.322
Phosphoenzyme	0.340	0.295	0.365

some change in the environment surrounding aromatic residues in phosphoenzyme at both pH's.

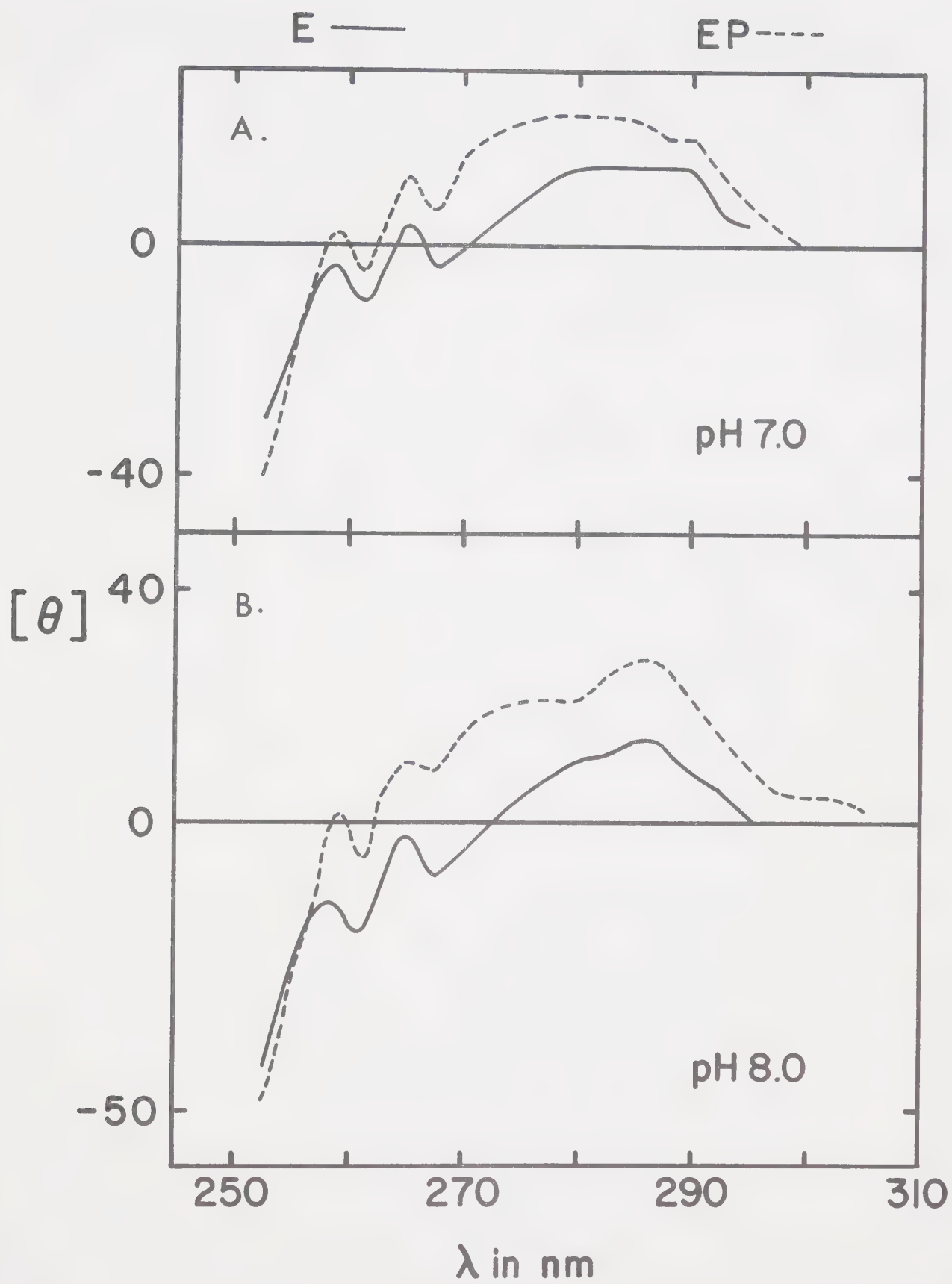
D. DISCUSSION

The improvements introduced to the purification procedure for PEP synthetase, primarily involving exploitation of charge differences between the phospho- and dephosphoenzyme, result in a reliable method for isolation of substantial quantities of apparently homogeneous enzyme. The specific activity of our preparations exceeds twice the value of that reported for previous preparations (13).

The availability of large quantities of pure enzyme has facilitated its physical characterization. We are confident that molecular weight estimates by gel filtration procedure (7) have been on the high side and that the correct value is near 150,000, representing a dimer of apparently equivalent subunits. The appearance of only one band following electrophoresis under denaturing conditions (Fig. 1) together with the number of peptides counted in the tryptic fingerprint (Fig. 4) indicates that if there are any differences in size and amino acid sequence of the subunits, they must be very small.

The increased stability of the dimer at low temperature indicates that hydrophobic interaction cannot be alone responsible for subunit interaction, since apolar interactions in aqueous system are known to be stabilized by increasing the temperature from 5° to 20° (68). The strength of interaction between the subunits is distinctly enhanced by phosphorylation of the enzyme, however, as shown by the greatly diminished tendency of the phosphoenzyme to dissociate at either temperature. Such an effect may be the result of a significant struc-

Figure 6. Near-ultraviolet circular dichroism spectra of PEP synthetase at pH 7.0 (A) and at pH 8.0 (B). (—) dephosphoenzyme, (---) phosphoenzyme.



tural rearrangement involving adjustment of atomic positions in the subunit contact domain. The results from circular dichroism experiments, which indicate structural change accompanying phosphorylation of the enzyme, also support the above argument.

CHAPTER IV
IDENTIFICATION OF PHOSPHOHISTIDINE IN THE
PHOSPHOENZYME INTERMEDIATE

A. INTRODUCTION

Although a phosphoenzyme form has been reported to be an obligatory intermediate for the reaction of PEP synthetase (7, 69), no attention has been focussed on the nature of this phosphoenzyme. Identification of phosphorylated amino acid residues in phosphoproteins provides insight into catalytic roles of such residues, the aspects of the protein structure which contribute in such a role, and give some clues about evolution or conservation among the enzymes that contain similar kinds of intermediate, with respect to their structure or catalytic mechanism. For example, from the studies of phosphorylated sites in substrates of protein kinases, it was found that of all 25 protein substrates compared there seemed to be a common feature in amino acid sequences surrounding sites phosphorylated by protein kinases (70). Of all the protein substrates studied, the serine residue is the phosphorylated site. In general, all phosphorylated sites are separated from either lysine or arginine by no more than two amino acids (70). This leads to some suspicion that this unique primary sequence plays a prominent role in the mechanism by which the protein kinases recognize their substrates. Among the enzymes that contain phosphohistidine, succinyl CoA synthetase and ATP-citrate lyase show many analogous counterparts in terms of mechanism of reactions (71). A phosphoprotein involved in the uptake and phosphorylation of hexoses in bacterial systems, formed by interaction with phosphoenolpyruvate, was shown

to contain phosphohistidine (26). Since PEP synthetase can be phosphorylated by PEP as well as by ATP, it would be of interest to determine whether the site of phosphorylation of this enzyme is similar to that of the transport protein.

As a guide to further experiments, treatment of the phosphoenzyme at different pH can provide some clues about the type of amino acid residue being phosphorylated. The presence of phosphohistidine would be indicated by its instability in acid pH; thus it could be identified by alkaline hydrolysis of phosphoprotein followed by separation and identification by comparison of its properties with authentic phosphohistidine (20). On the other hand, the instability of phosphoprotein in alkaline pH would suggest the presence of phosphorylated serine or threonine which can be identified after acid hydrolysis (72). However, there is some difficulty about identification of glutamylphosphate or aspartylphosphate, which are unstable in both acid and alkaline pH. A commonly used method involves reaction with hydroxylamine and subsequent Lossen rearrangement and isolation of its product after acid hydrolysis (73). Recently, Digani and Boyer (30) reported a simplified method for identification of acylphosphate linkage in proteins. Their method involves reductive cleavage of the acylphosphate bond with [^3H]-borohydride to form a labeled aminohydroxy acid residue, followed by acid hydrolysis and identification of the radioactive ω -hydroxyamino acid formed from the phosphorylated amino acid residue.

This chapter describes the studies attempted to identify the site of phosphorylation of PEP synthetase. The results obtained from various approaches indicate the presence of phosphohistidine in the phosphoenzyme intermediate.

B. METHODS

1. Effect of pH on stability of phosphoenzyme

The labelled phosphoenzyme was incubated for 1 hour at 25° with 0.1 M buffers of pH 0.5 to 13.5. The following buffer systems were employed: pH 0.5, 1 N HCl; pH 1.09 and 2.5, KCl-HCl; pH 4.35, 5.05 and 5.95, Tris-maleate; pH 6.0, 6.80 and 7.5, phosphate; pH 8.0, Tris HCl; pH 9.6 and 10.2, KHCO_3 -KOH; pH 11.1, glycine-KOH; pH 12.9, 0.2 N KOH; and pH 13.5, 1 N KOH. Controls in water were kept at 0° during incubation. After incubation, the mixtures were immediately chilled in an ice-bath and neutralized. The protein was precipitated by addition of cold perchloric acid to final concentration of 0.2 N. The precipitate was collected by filtration on a membrane with 5 μ pore size and was washed several times with cold 0.2 N perchloric acid. The membrane was air-dried, and counted for radioactivity in Scintiverse scintillation cocktail.

2. Hydroxylamine treatment

Neutralized hydroxylamine solution was prepared by mixing equal volumes of 4 M KOH and 4 M hydroxylamine hydrochloride just before use. ^{32}P -Phosphoenzyme was treated with 0.2 N hydroxylamine at 25°, aliquots were taken at various time intervals and the protein-bound radioactivity was determined as described above.

3. Hydrolysis in HCl solution

The hydrolysis of $\text{E-}^{32}\text{P}$ was carried out in HCl solutions ranging in concentration from 0.1 N to 2.5 N at 48°. A similar technique as for hydroxylamine treatment was used for measurement of the rate of acid hydrolysis.

4. Borohydride reduction

i) About 3 mg of phosphoenzyme was dissolved in 0.7 ml of dimethylsulfoxide, and 0.3 ml of [^3H]- NaBH_4 solution (18 mM) containing 15 mCi per ml was added. The reaction mixture was incubated at room temperature for 15 min and the reaction was stopped by adding 10 ml of cold 0.44 M perchloric acid, followed by centrifugation. The precipitate was washed 3 times with cold 0.4 M perchloric acid. A control sample, using dephosphorylated enzyme, was treated identically. The precipitates of reduced phosphorylated protein and the control companion were hydrolysed in 6 N HCl at 110° for 22 hours. The hydrolysates were evaporated to dryness under vacuum. The residue was taken up with 0.5 ml of water and dried twice in order to completely remove exchangeable tritium. Finally, it was dissolved in 0.2 ml of water. Aliquots (20 μl) were taken for high voltage electrophoresis.

ii) The reaction mixture contained 0.5 mg of enzyme in 0.1 M Tris-HCl, pH 6.8. The phosphoenzyme or diphosphoenzyme was treated at 0° with four 0.01 ml portions of 1 M NaBH_4 added alternately with four 0.005 ml portions of 1 M acetic acid to maintain the pH over a period of 20 min, according to Grazi et al. (74). The reaction mixture was then warmed to room temperature and passed through a column of Sephadex G-50 to remove excess reagent. The enzyme activity was then assayed.

5. Alkaline hydrolysis of phosphoenzyme and chromatography of its hydrolysate

The ^{32}P -enzyme was hydrolysed in a sealed tube with 3 N NaOH at 110° for 100 min. After cooling, the tube was opened and the contents were diluted approximately 50-fold with water and applied to a 2 x 15 cm Dowex-1-(OH) column. The column was washed extensively with water and

then eluted with linear gradient from 0 - 1.0 M KHCO_3 pH 8.3 (1 litre total).

C. RESULTS

1. Phosphorylation and dephosphorylation of PEP synthetase

When the enzyme was incubated with 1 mM ATP- β - ^{32}P and 1 mM MgCl_2 at 25° for 20 min, it was found that 1 mole of phosphate was associated with 1 mole of enzyme (mol. wt. 150,000). There was no further phosphorylation when the incubation time was prolonged to 1 hour.

Consistent with the concept that the phosphoenzyme is an obligatory intermediate of the PEP synthetase reaction, we have found that pyruvate, which is the second substrate of reaction, has the ability to dephosphorylate the enzyme. Results shown in Fig. 7A indicate that pyruvate, in the presence of Mg^{2+} , dephosphorylates the enzyme very quickly so that equilibrium is reached in seconds. If the phosphoenzyme is incubated with pyruvate alone, the rate of dephosphorylation was much slower than in the presence of Mg^{2+} (Fig. 7B). These results agree well with those reported by Berman and Cohn (13) who implicated Mg^{2+} ion in the binding of pyruvate to the enzyme.

2. Identification of phosphorylated amino acid residue

As a preliminary guide to the identification of the site of phosphorylation of PEP synthetase, the stability of the phosphoenzyme under various conditions was studied. The effect of pH on the rate of hydrolysis of the phosphoenzyme was investigated and the results are given in Fig. 8. Hydrolysis of the phosphoryl-enzyme bond was very slow near neutrality and at alkaline pH, but it was greatly accelerated on decreasing the pH. This extreme acid lability eliminated the possibility

Figure 7. Dephosphorylation of PEP synthetase in presence of pyruvate and MgCl_2 . [^{32}P] enzyme was incubated with 0.5 mM pyruvate and 5 mM MgCl_2 (A) or with 1 mM pyruvate (B). Samples were withdrawn at timed intervals and protein-bound radioactivity was measured as described in the text.

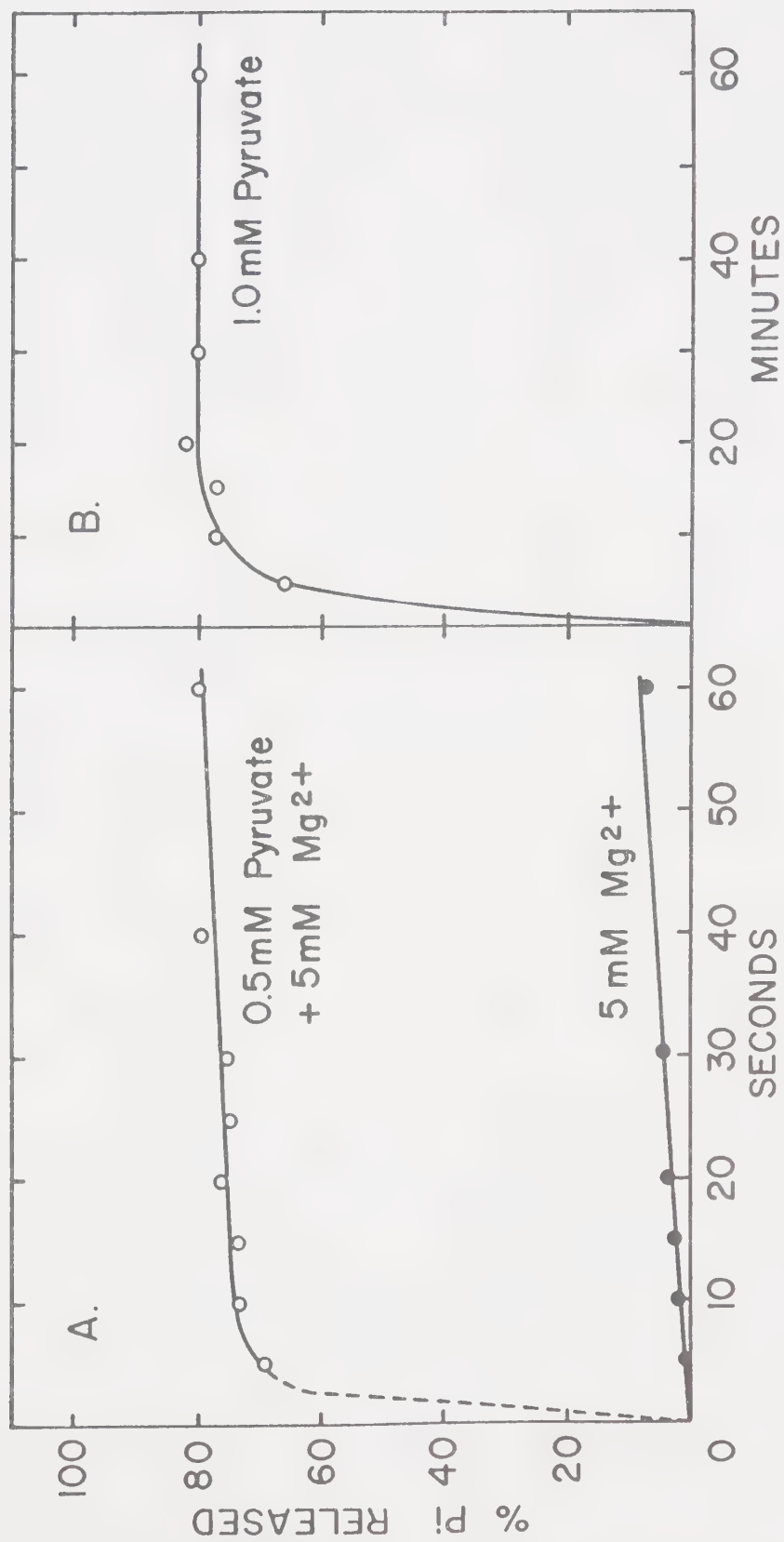
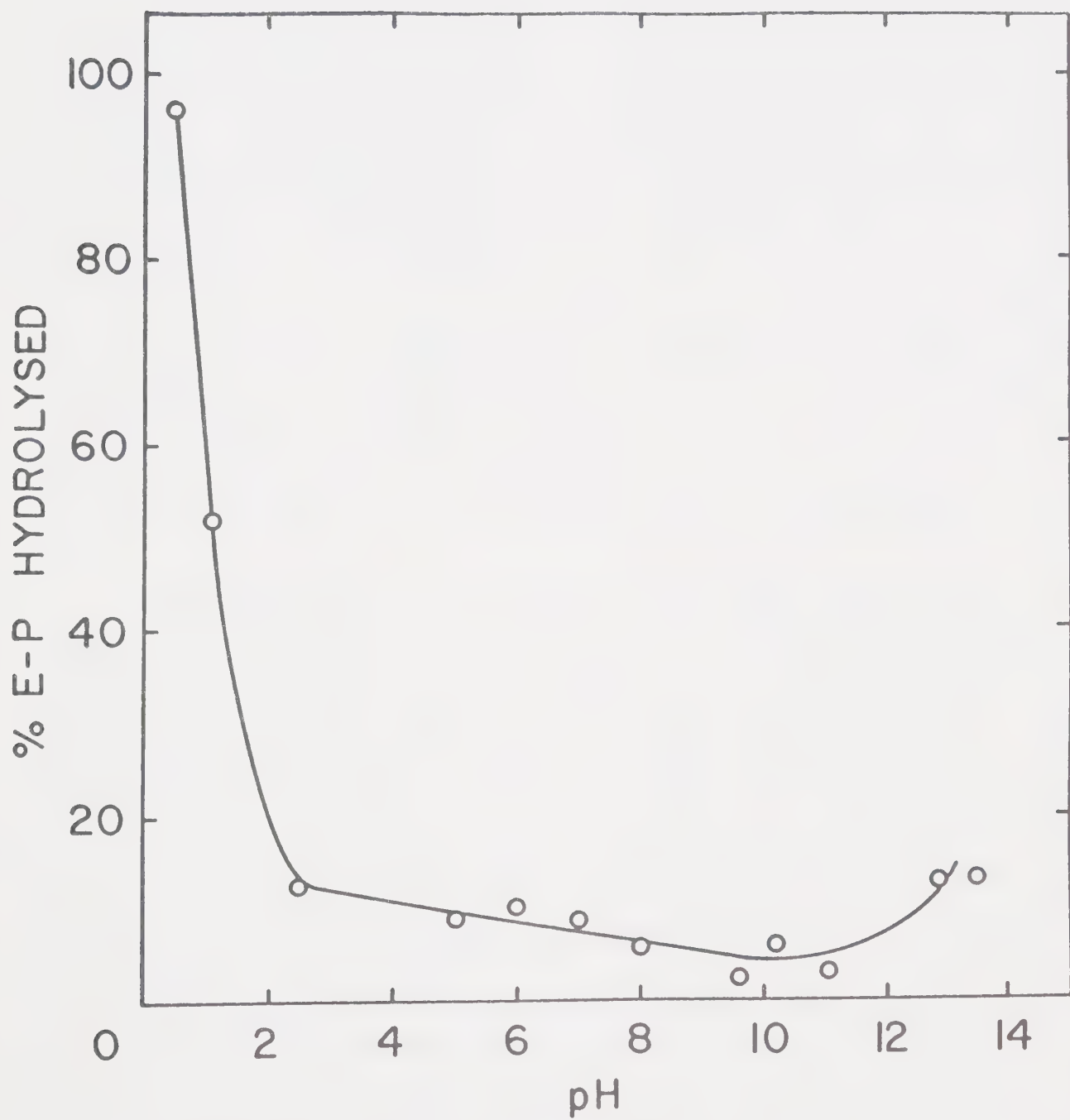


Figure 8. Effect of pH on the rate of hydrolytic release of $^{32}\text{P}_i$ from $[\text{}^{32}\text{P}]$ -PEP synthetase. The labelled enzyme was incubated at 25° for one hr at the indicated pH. Other details are given in the text.



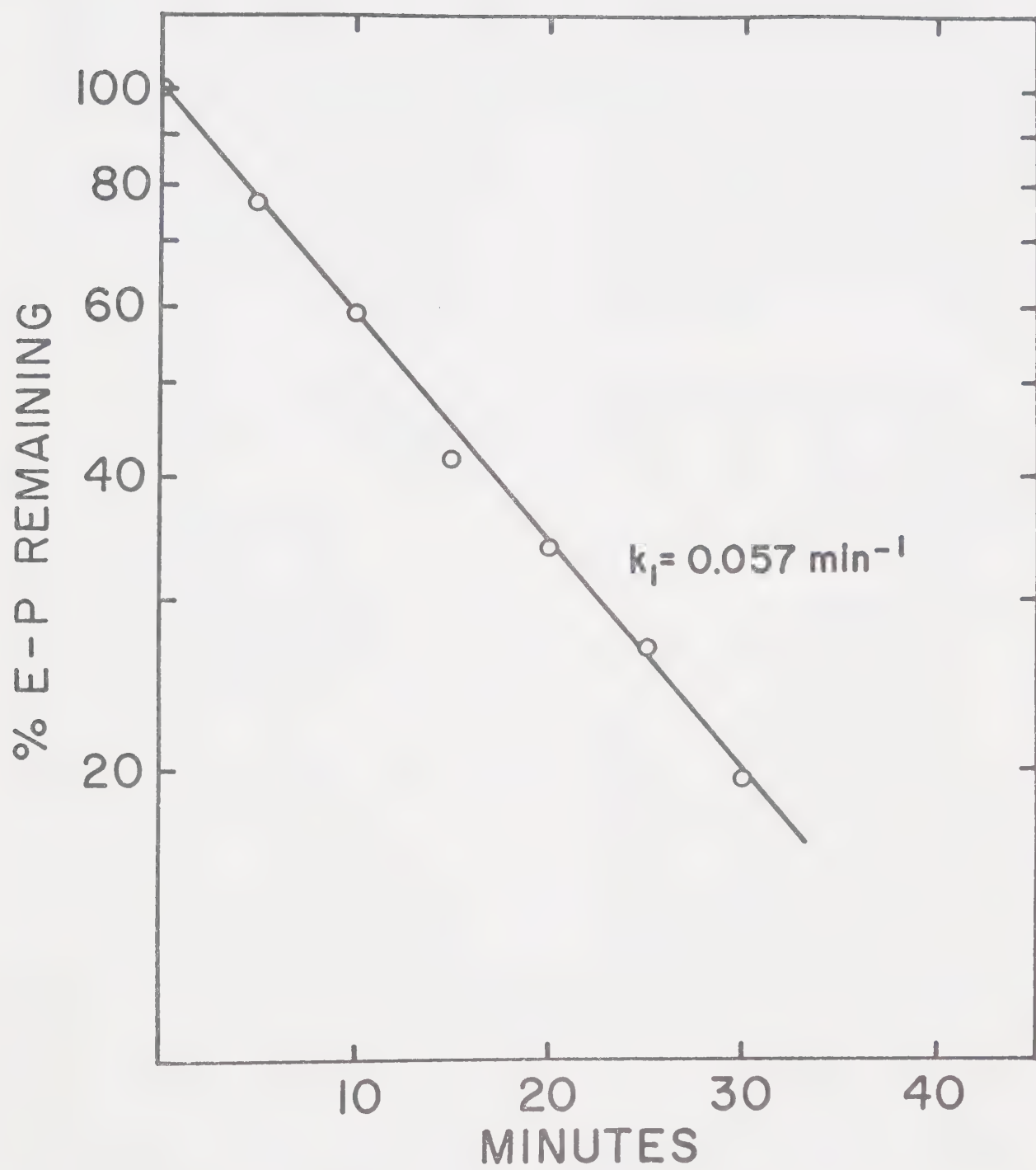
that the phosphorylation had occurred on a serine residue. Acylphosphates or phosphohistidine, on the other hand, are known to be acid labile (71, 75).

If the phosphoryl group were attached to the enzyme by means of an acyl phosphate linkage to a glutamyl or aspartyl residue, it would be expected to be rapidly released by the treatment with hydroxylamine accompanied by hydroxamate formation at the carboxyl group of the acyl residue. When the [^{32}P]-phosphoenzyme was subjected to treatment with 0.2 M hydroxylamine, pH 7.2, ^{32}Pi was released in a slow reaction obeying pseudo-first order kinetics (Fig. 9). The second order rate constant for the process was calculated to be $0.29 \text{ min}^{-1} \text{ M}^{-1}$, a value at least two orders of magnitude lower than expected for reaction of hydroxylamine with enzymatic acyl phosphate under the same conditions (31).

Additional evidence against the presence of acyl phosphate in the phosphoenzyme was obtained by application of the method of Degani and Boyer (30) for identification of such residues. This technique depends upon the reductive cleavage of the phosphoenzyme with [^3H]-borohydride followed by acid hydrolysis and identification of ω -hydroxy-amino acid from the hydrolysate. When the technique was applied to PEP synthetase, there was no significant incorporation of radioactivity into the phosphoenzyme as compared to the dephosphoenzyme. No tritium was detected in homoserine, homoserine lactone or α -amino- δ -hydroxyvaleric acid (the products expected for reductive cleavage of aspartylphosphate and glutamylphosphate residues) following paper electrophoresis of the hydrolysate.

Finally, the presence of acyl phosphate was ruled out by testing

Figure 9. Effect of hydroxylamine on the rate of release of $^{32}\text{P}_i$ from [^{32}P]-PEP synthetase. The labelled enzyme was incubated with 0.2 N hydroxylamine (pH 7.4) at 25°. Samples were withdrawn at timed intervals and protein-bound radioactivity was measured as described in the text.



the activity of the enzyme recovered from either hydroxylamine or borohydride treatment. Had these reagents brought about cleavage of a catalytically important acylphosphate, the resulting enzyme derivative would be expected to be inactive with the putative carboxyl converted to either its corresponding hydroxamate or primary alcohol, respectively. Results presented in Table V indicate that the phosphoenzyme retained full activity after hydroxylamine cleavage or borohydride treatment.

3. Identification of phosphohistidine in hydrolysate of phosphoenzyme

Following procedures developed for other phosphohistidine-containing proteins (20, 76), [^{32}P]-phosphoenzyme was hydrolyzed in 3 N NaOH for 100 min at 110° and the products were separated by anion exchange chromatography (Fig. 10). The first radioactive peak was identified as inorganic phosphate and the position of the second peak, containing about 30% of the starting radioactivity, agreed well with that expected for phosphohistidine (20). The pooled fractions were then applied to a second Dowex-1-OH column; one radioactive peak was eluted with 1 M ammonium carbonate. A sample from this peak was subjected to high voltage electrophoresis in 1% ammonium carbonate, pH 9.0. As shown in Fig. 11, two radioactive spots were detected, one corresponding to inorganic phosphate and the other to the position of authentic 3-phosphohistidine. Similar results, with approximately 50% hydrolysis of phosphohistidine occurring during high voltage electrophoresis under these conditions have been obtained by Wang et al. (77) for the case of succinyl CoA synthetase, and enzyme known to contain phosphohistidine.

In spite of these observations, some doubt persisted about the nature of the catalytically active residue, since the possibility

TABLE V

Retention of Activity Following Hydroxylamine or Borohydride
Treatment of Phosphoenzyme

Condition	Per cent activity
Hydroxylamine treatment:	
1. E-P before treatment (1 phosphoryl/ enzyme)	100
2. E-P + 0.2 N hydroxylamine, pH 7, 2 hr (<0.01 phosphoryl/enzyme)	100
Borohydride reduction:	
1. E-P + borohydride	96
2. E + borohydride	99
3. E + borohydride, ATP, Mg^{2+}	101

Figure 10. Anion exchange chromatography of alkaline hydrolysate of [^{32}P]-PEP synthetase.

5 mg of labelled enzyme (4.5×10^5 cpm) was digested in 3 N NaOH at 110° for 100 min and the hydrolysate was applied to a column of Dowex-1 (OH^-) as described in the text. Peak I was identified as P_i and contained about 70% of the radioactivity. Peak II was subjected to high voltage electrophoresis (see text and Fig. 11).

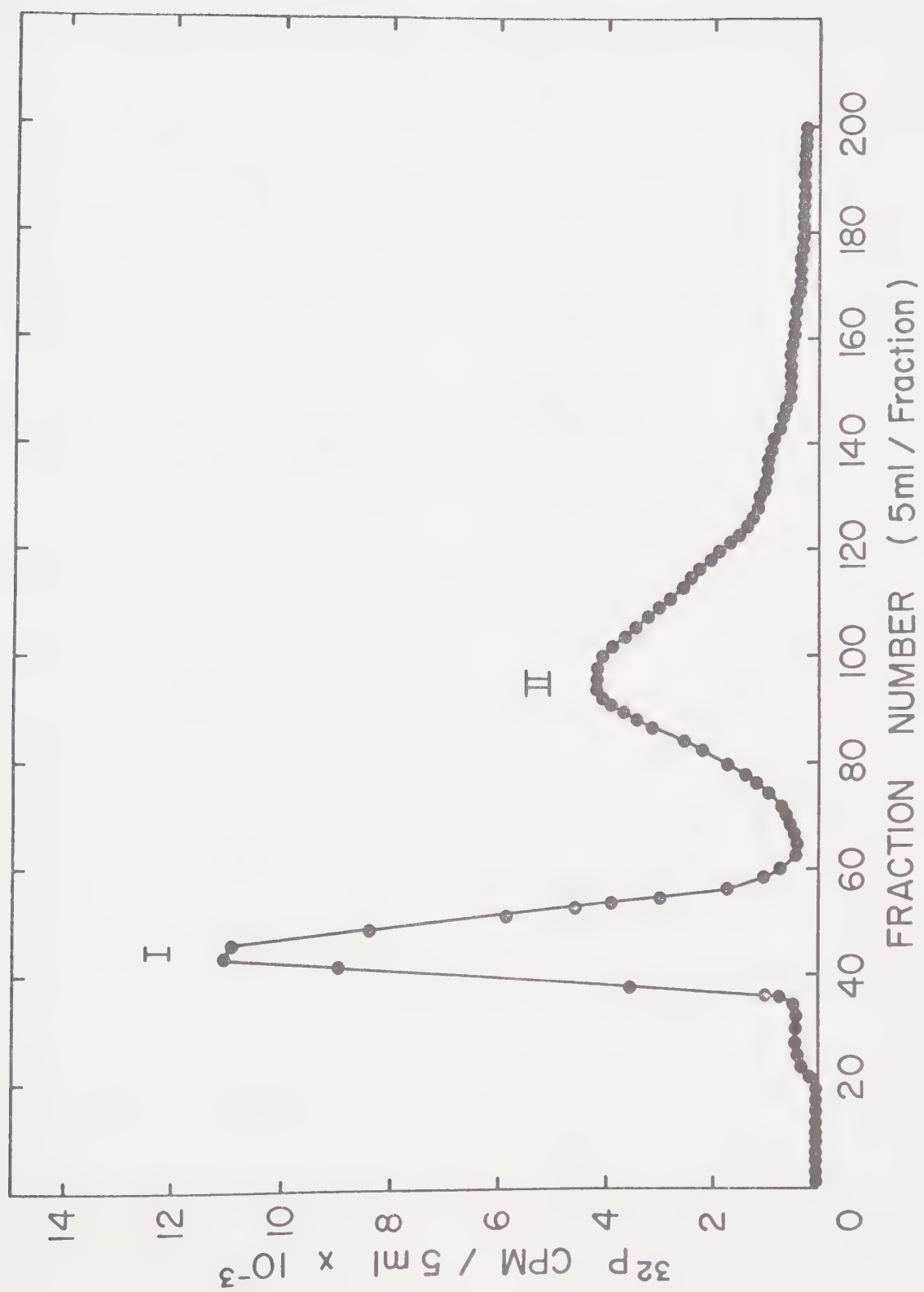
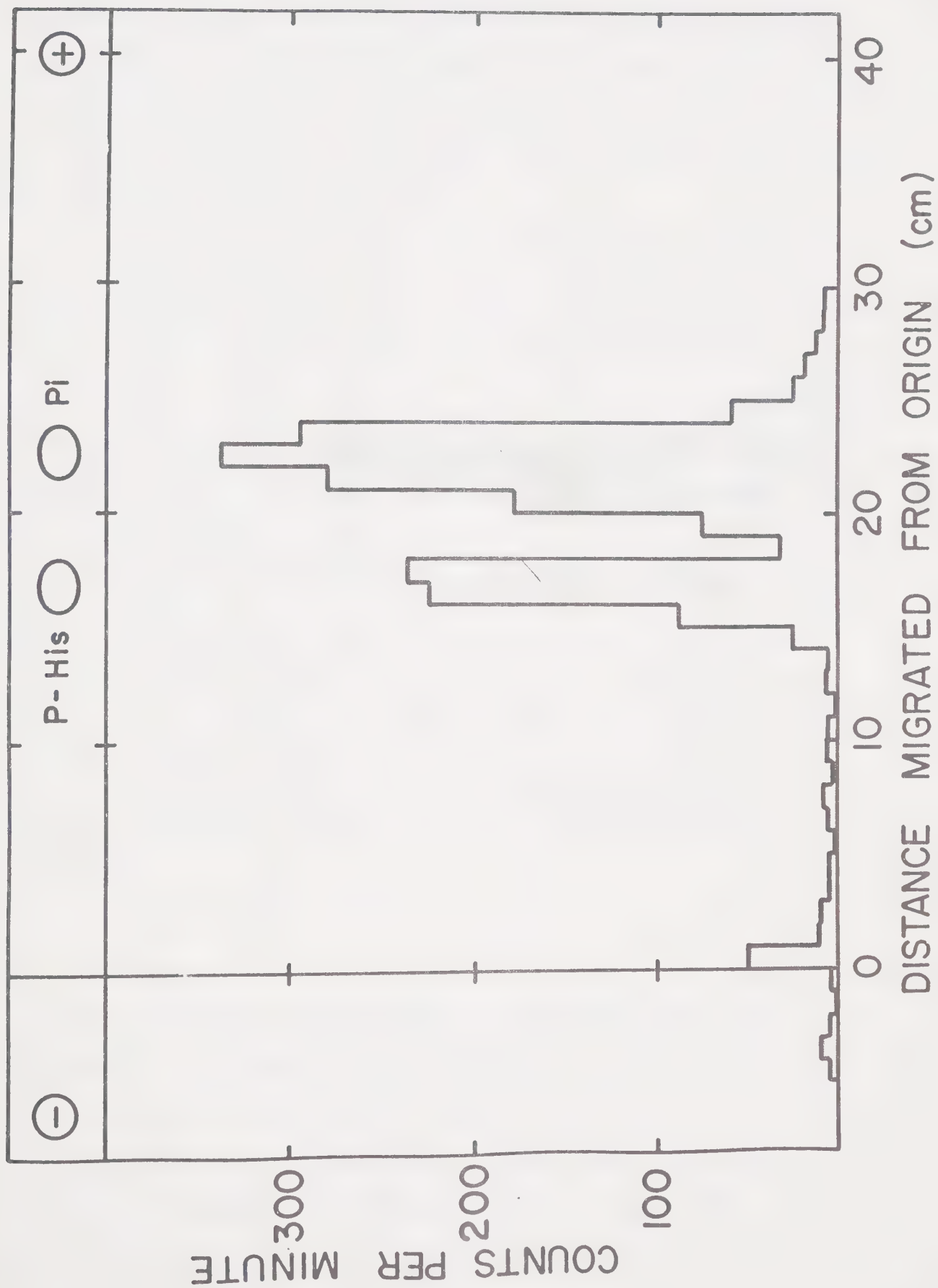


Figure 11. High voltage electrophoresis (pH 9.0) of peak II fraction from Figure 10. Pooled fractions, (nos. 90 - 105 from Fig. 10) were applied to a 1 x 15 cm column of Dowex-1 (OH^-). Elution with 1 M ammonium carbonate yielded one radioactive peak, a portion of which was lyophilized, taken up in water and subjected to high voltage electrophoresis together with authentic 3-phosphohistidine. Histidine-containing spots were located with Pauly reagent. The paper was cut into 1 cm strips and radioactivity was counted by liquid scintillation.



remained that alkaline hydrolysis of phosphorylated enzyme was accompanied by migration of the phosphoryl group to a histidine residue from a primary site of phosphorylation. Such migration has been proposed to account for difficulties in establishing the nature of the phosphorylated residue in ATP-citrate lyase (21, 73).

Final confirmation of the identification of phosphohistidine in the phosphorylated derivative of PEP synthetase was then made by a study of the kinetics of hydrolysis of the phosphoenzyme in hydrochloric acid solution. Fig. 12 shows that at all hydrogen ion concentrations studied, the plots of logarithm of residual ^{32}P -protein versus time are linear, indicating that only one species of phosphoryl bond is hydrolysed. Fig. 13 shows the pseudo-first order rate constant for the hydrolysis of Pi from phosphoenzyme as a function of the normality of acid. Also shown are the rate constants obtained for the hydrolysis of the 3-phosphohistidyl residue of succinyl CoA synthetase (78). The excellent agreement of the data for these two enzymes, together with the above identification of phosphohistidine in the protein hydrolysate, leaves little doubt that a histidine residue is the site of phosphorylation of PEP synthetase.

D. DISCUSSION

The stoichiometry of phosphorylation of the enzyme which we have determined is in agreement with values reported by Cooper and Kornberg (7) and Berman and Cohn (13), who reported, respectively, 1.0 and 1.5 phosphoryls bound per 250,000 molecular weight. When these values are corrected to correspond to a molecular weight of 150,000, they become 0.6 and 0.9 phosphoryls/mole, consistent with our data which have been

Figure 12. Acid hydrolysis of [^{32}P]-PEP synthetase. Labelled enzyme was incubated at 48° in HCl solution of indicated concentration.

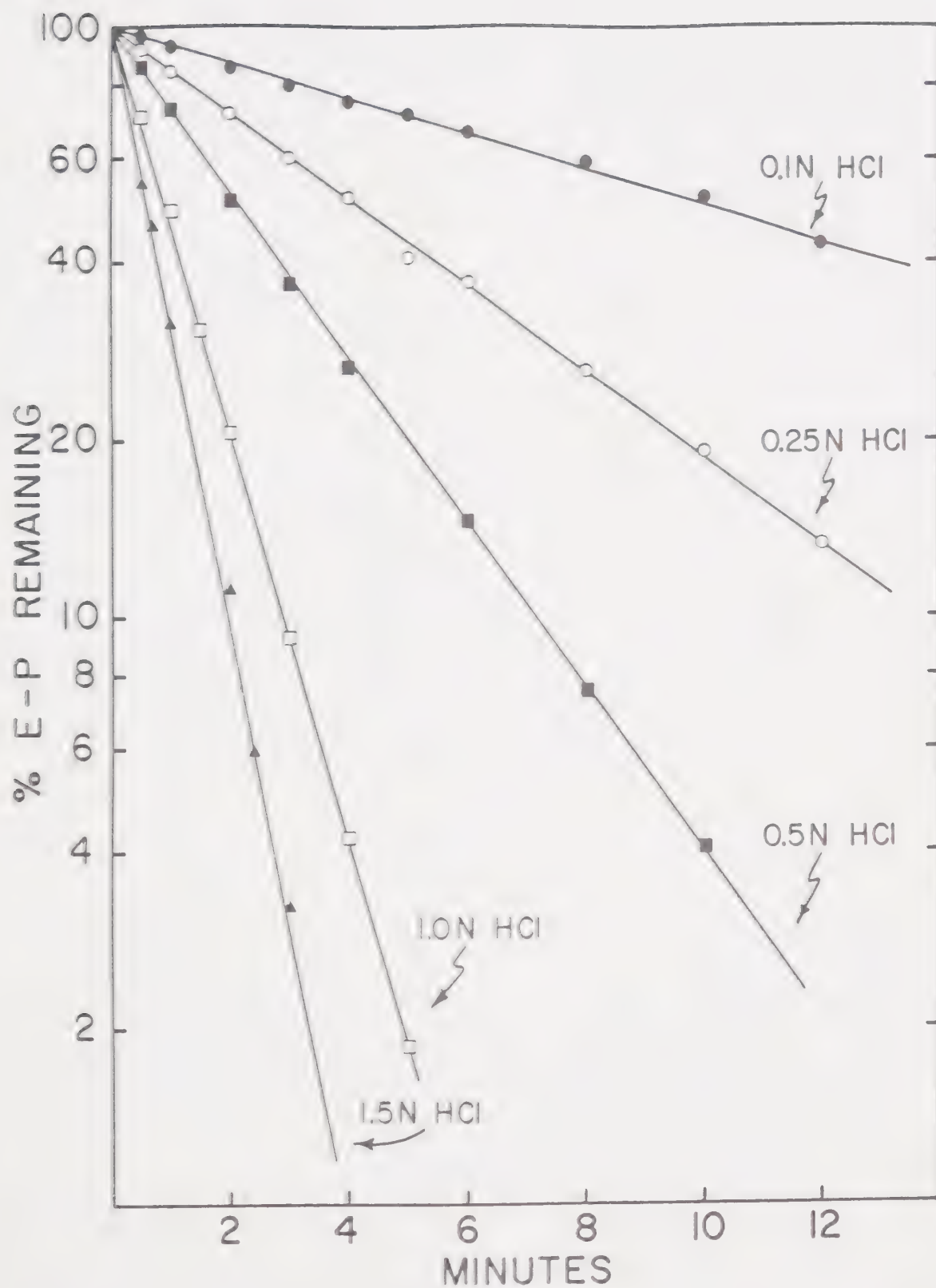
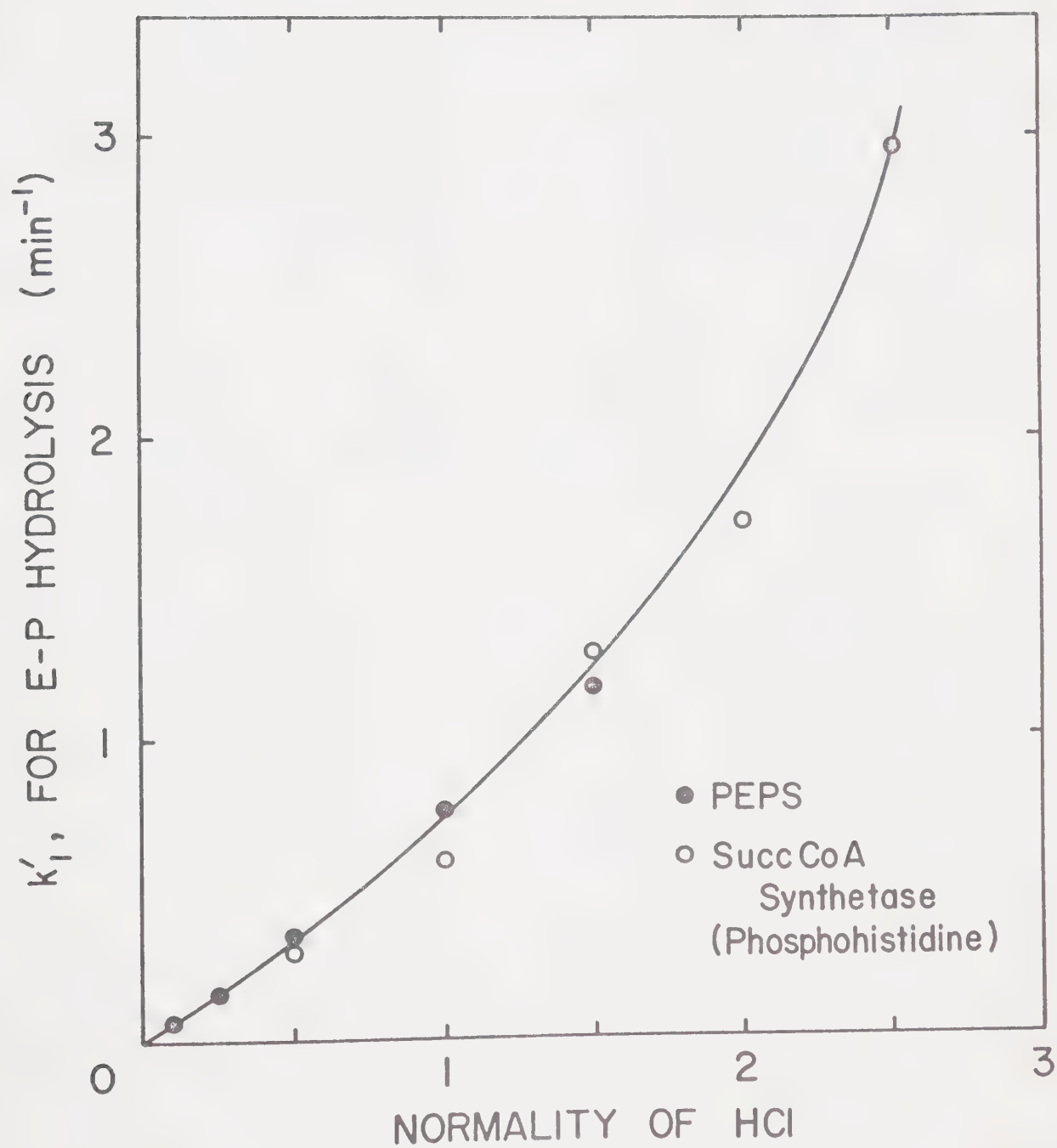


Figure 13. Effect of normality of acid on the pseudo-first order rate constant (k'_1) for [^{32}P]-PEP synthetase hydrolysis.

●—●, rate constants for hydrolysis of PEP synthetase (data obtained from Fig. 13). O—O, rate constants for hydrolysis of [^{32}P]succinyl CoA synthetase, known to contain 3-phosphohistidine (data obtained from Wang, T.T. (



obtained with an enzyme preparation of improved specific activity. From the sedimentation equilibrium studies reported in Chapter III, it has been found that the strength of interaction between the subunits is enhanced by phosphorylation of the enzyme. It is noteworthy that phosphorylation of only one of the two subunits has occurred to produce the stable dimer. The proposed structural rearrangement accompanying monophosphorylation may therefore also cause alteration in the sister active site, thus making its phosphorylation less favorable or impossible and accounting for the apparent half-sites reactivity. It is particularly noteworthy in this context that succinyl CoA synthetase, another phosphohistidine-containing enzyme showing half-sites behaviour, also undergoes a large conformational change upon monophosphorylation (79).

We have considered the possibility that the apparent half-sites behaviour may be the simple consequence of the presence of dissimilar subunits. While we cannot completely rule out this alternative, the appearance of only one band following electrophoresis under denaturing conditions (Chapter III, Fig. 1) together with the number of peptides counted in the tryptic fingerprint (Chapter III, Fig. 4) indicates that any differences in size and amino acid sequence of the subunits must be very small.

The presence of phosphohistidine in the phosphoenzyme has been established by its identification in alkaline digests (Fig. 11) and by measurements of the rate of acid hydrolysis of E-P (Fig. 13). The slow reactivity of the phosphoenzyme toward hydroxylamine has not been reported to be characteristic of phosphohistidine, however (76). Similar results have been obtained by others in experiments designed to identify the site of phosphorylation of ATP-citrate lyase. While Cottam

and Srere (21) have identified phosphohistidine in alkaline digests of the phosphorylated derivative of that enzyme, Suzuki et al. (73) reported identification of the product expected for a glutamyl phosphate residue following hydroxylamine cleavage and Lossen rearrangement. It has been suggested (71) that phosphoryl migration may account for the latter results. A similar explanation could be offered for the release of phosphate from PEP synthetase in the presence of hydroxylamine, but the fact that such treatment does not affect the catalytic activity of the enzyme effectively rules out a mechanism involving acyl phosphate. More likely, enhanced base-lability of phosphohistidine in its particular environment at the active site could account for the result.

The sequence of phosphohistidine-containing peptides derived from succinyl CoA synthetase (77) and phosphoglycerate mutase (80) have been determined. They do not resemble each other, but surprisingly, the sequence of the peptide from phosphoglycerate mutase was found to be homologous to a portion of the nucleotide-binding domain of lactate dehydrogenase (81). It will be of interest to determine whether the sequence surrounding the phosphohistidine residue of PEP synthetase resembles either of these. Homology seems probable, however, between PEP synthetase and a similar enzyme from Propionibacter shermanii, pyruvate, phosphate dikinase (E.C. 2.7.91). The latter enzyme, which catalyses reaction:



has been estimated to have a molecular weight of approximately 150,000 (11). It has recently been suggested that this enzyme may also be a dimer showing half-sites behaviour with respect to its phosphorylation

(82). Phosphohistidine has been found in alkaline digests of its phosphoenzyme form (27). Detailed structural comparisons of PEP synthetase and pyruvate phosphate dikinase may provide insight into the process of enzyme evolution.

CHAPTER V

EFFECT OF SOME METABOLITES AND METAL IONS ON ACTIVITY OF PEP SYNTHETASE

A. INTRODUCTION

The control of activity of PEP synthetase has been investigated by various authors (14, 46). So far, it has been found that the enzyme is controlled by its end-products (AMP and PEP) (14, 46) and by energy charge (14). We consider that it is necessary to examine the strategy employed by the cells to manage the problems of metabolic traffic which arise when they switch over from growth conditions under which glycolysis predominates to those under which gluconeogenesis is essential (i.e., growth on C_3 or C_4 compounds). Glycolysis shares with gluconeogenesis the series of freely reversible, constitutive enzymes producing FDP from PEP. For gluconeogenesis to occur, the bacterial cells induce the production of enzyme PEP synthetase when a C_3 compound (alanine, pyruvate or lactate) is the energy source. In this case, PEP is produced at the expense of ATP. However, since the cells also contain pyruvate kinase, PEP produced by PEP synthetase could easily be reconverted to pyruvate and ATP instead of giving rise to triose phosphate. Thus, the simultaneous functioning of both of these enzymes during active gluconeogenesis would be deleterious to the organisms if these enzymes are left uncontrolled. This would be wasteful not only because the synthesis of hexoses would be curtailed but also because there would be a net hydrolysis of ATP by the futile cycle.

There are two types of pyruvate kinase in E. coli. One is activated by F-1,6- P_2 and is an inducible enzyme during active glycolysis (83) and the other is activated by AMP and ribose-5-P (84). (This chapter

reports the studies of the effect of these effectors of pyruvate kinase in the hope that they may have reciprocal effects on PEP synthetase.) The effect of other metabolites such as aromatic amino acids which require PEP as the precursor were also studied. So far we have failed to demonstrate any prominent effect of these metabolites on PEP synthetase.

Various enzymes that use pyruvate or PEP as substrate have been reported to be inhibited by oxalate. As also reported in this chapter, PEP synthetase is not exceptional, being strongly inhibited by oxalate which is competitive with pyruvate.

The inhibition effect of some divalent metal ions on PEP synthetase activity is also reported.

B. METHODS

The assay of the enzyme activity is essentially as described by Cooper and Kornberg (46). The reactions were performed at 30°.

1. Forward reaction

For assay under saturating conditions, the reaction mixture (0.5 ml) consisted of 2 mM pyruvate, 10 mM ATP, 10 mM MgCl_2 , 0.1 M Tris-HCl (pH 8.0) and the appropriate amount of PEP synthetase. Aliquots of 0.1 ml were withdrawn at 1 min intervals and allowed to react with dinitrophenylhydrazine (0.3 ml of 0.1% reagent in 2 N HCl plus 0.9 ml H_2O) for 10 min. The concentration of unreacted pyruvate was determined, after addition of 1.67 ml of 10% NaOH, by measuring the absorbance at 445 nm. Under these conditions, 1 μmole of pyruvate has an extinction of 6 absorbance units.

2. Reverse reaction

The phosphate-dependent formation of pyruvate from PEP and AMP was measured by coupling the reaction to lactate dehydrogenase. The reaction mixture (1.0 ml) consists of 0.1 M K phosphate buffer (pH 6.8), 5 mM MgCl_2 , 1 mM AMP, 1 mM PEP, 0.1 mM NADH and 10 units of lactate dehydrogenase. The reaction was started by adding an appropriate amount of PEP synthetase and the progress of the reaction was followed by measuring the decrease in absorbance at 340 nm using a Cary 15 spectrophotometer.

C. RESULTS

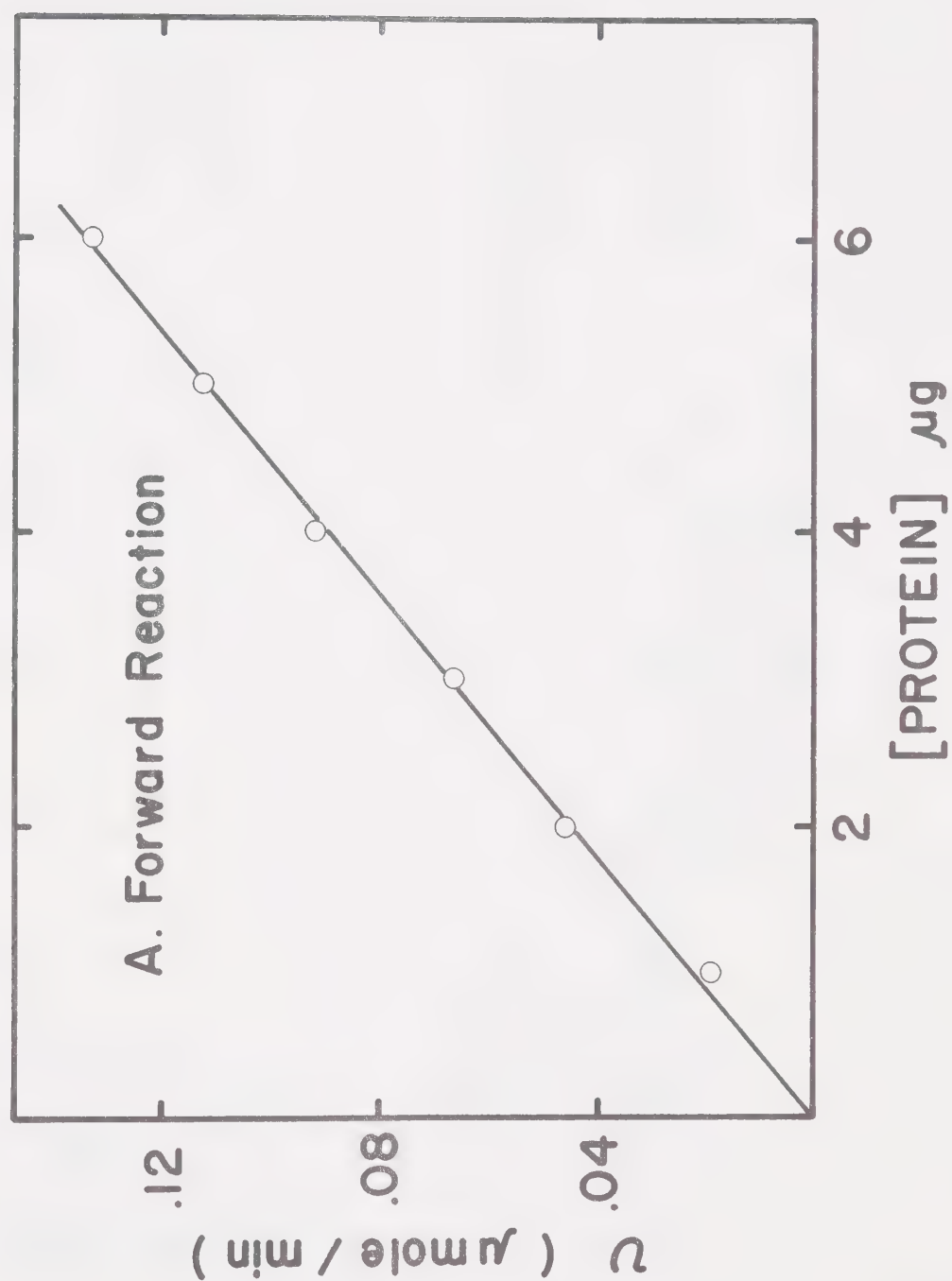
1. Some kinetic parameters of PEP synthetase

A difference in the apparent behavior of PEP synthetase in the forward and the reverse reactions was noted, namely in the dependence of its velocity on protein concentration. For the forward reaction the reaction velocity is directly proportional to protein concentration, whereas in the reverse reaction the plot of velocity against enzyme concentration shows a sigmoidal curve (Fig. 14). For assay of the unfavorable reverse reaction, at least 20 times the amount of enzyme used in the forward reaction is necessary to obtain a measurable reaction.

The plots of enzyme velocity against concentration of phosphorylated substrates, i.e., ATP and PEP, show the unusual characteristic of intermediary plateau regions (Fig. 15). For the reverse reaction this effect is more prominent when PEP is the variable substrate. These results suggest negative cooperativity of PEP synthetase with these substrates. However, the plots of velocity against [pyruvate] or [AMP] show normal hyperbolic curves. The apparent K_m of these reactants is

Figure 14. Effect of enzyme concentration on rate of reaction of PEP synthetase. A. Forward reaction (direction of PEP synthesis). B. Reverse reaction.

Effect of Enzyme Concentration



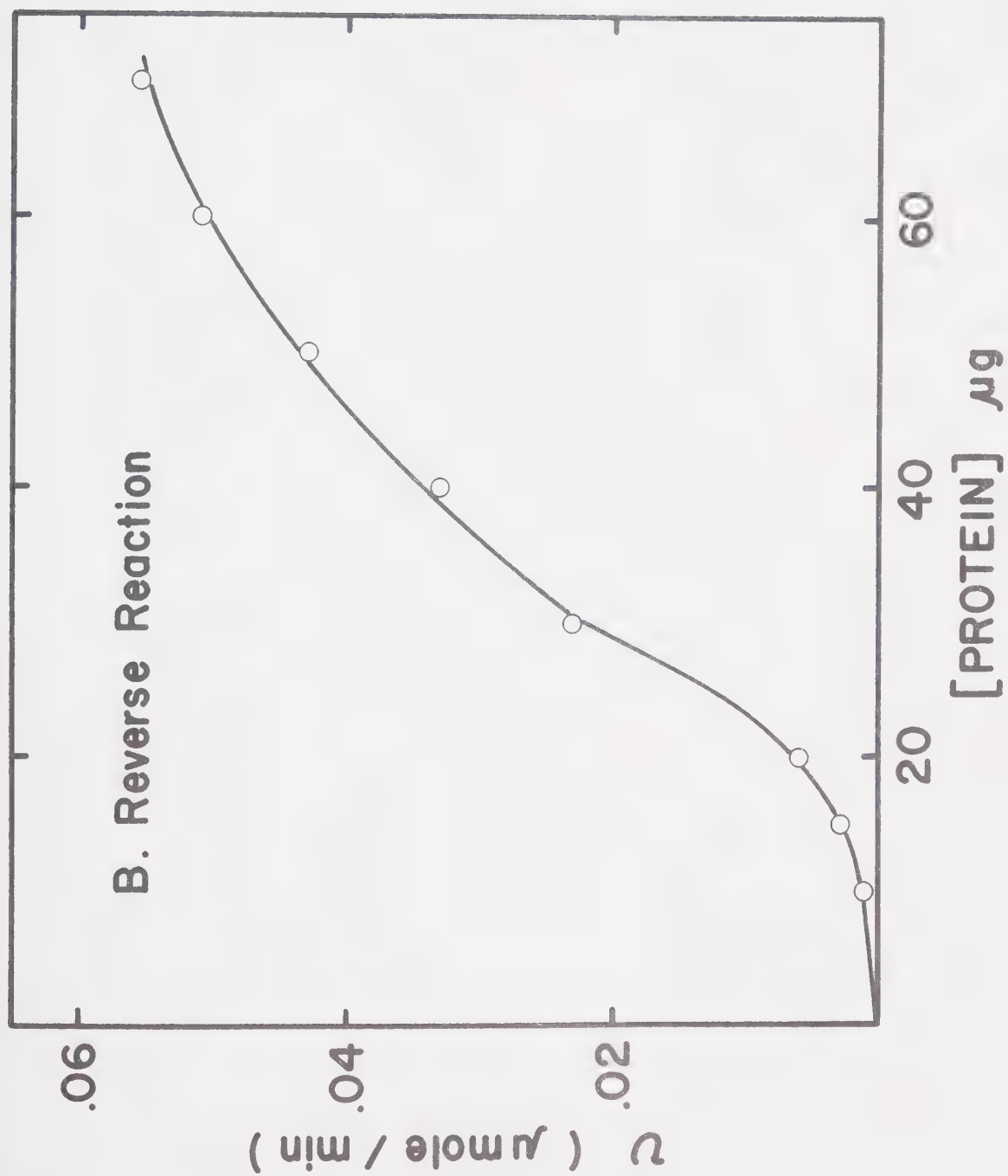
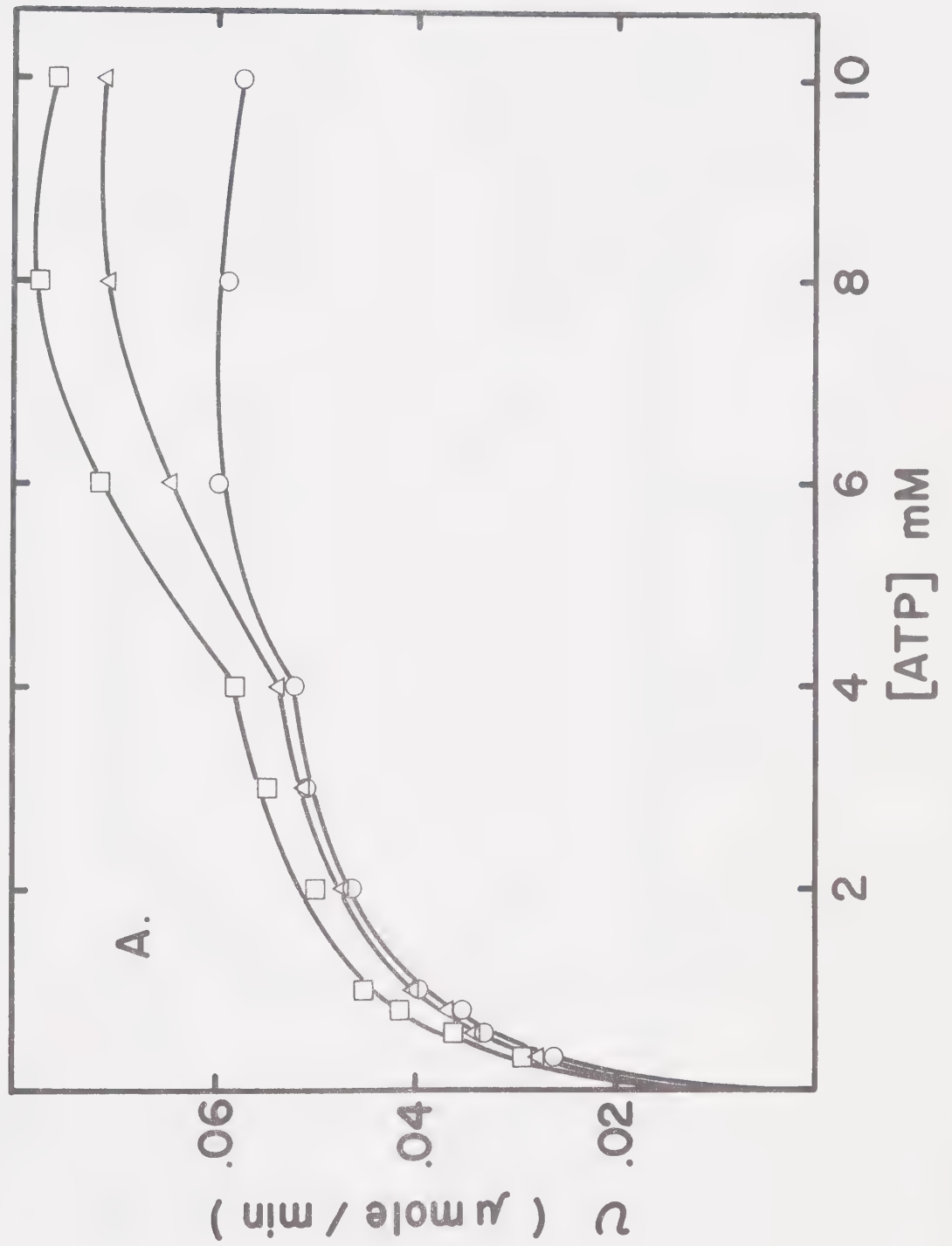
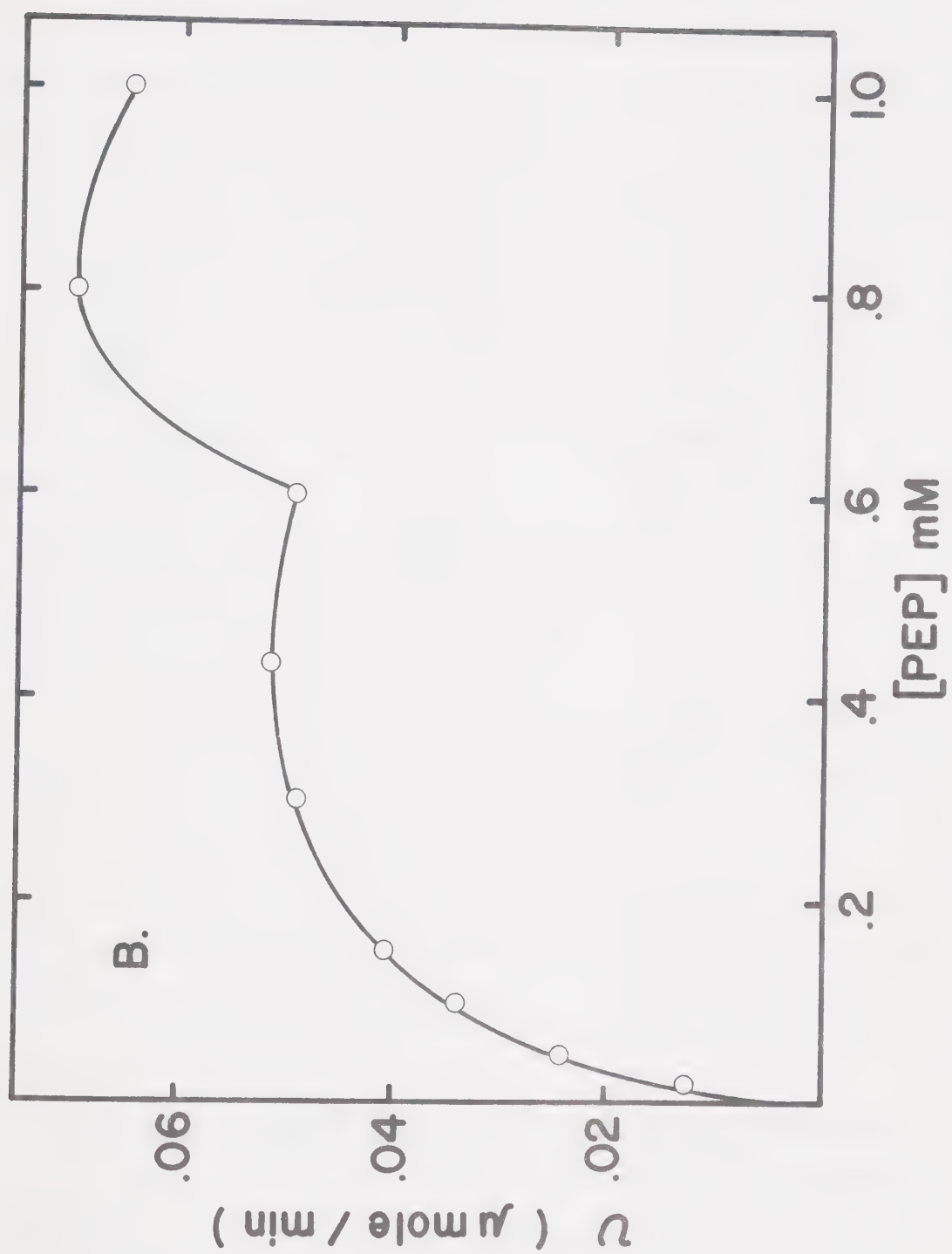


Figure 15. Effect of substrate concentration on rate of reaction of PEP synthetase. A. ATP is variable substrate. 0—0, at pH 7.4; □—□, at pH 8.0; Δ—Δ, at pH 8.4. B. PEP is variable substrate.

Plot of Velocity vs Substrate Concentration





shown in Table VI.

2. Effect of some metabolites on the enzyme activity

Ribose-5-phosphate and fructose-1,6-diphosphate, both of which are the activators of pyruvate kinase in E. coli (83, 84), were investigated for their effects on the activity of PEP synthetase. There does not seem to be any prominent effect of these metabolites on PEP synthetase. F-1,6-P₂ at 1 mM or 4 mM does not affect the enzyme activity when pyruvate is the variable substrate. Only when its concentration is increased to 10 mM does it cause slight inhibition of the reaction at low concentrations of pyruvate (Fig. 16A). However, when ATP is the variable substrate, 10 mM F-1,6-P₂ is virtually without effect on enzyme activity (Fig. 16B). The inhibitory effect of F-1,6-P₂ on PEP synthetase may not have any physiological significance, however, because the concentration used is very high.

Ribose-5-phosphate at concentration ranges from 0.5 mM to 10 mM does not have any effect on enzyme activity when either pyruvate or ATP is the variable substrate.

The effects of other metabolites that have been reported to affect pyruvate kinase are listed in Table VII. Citrate, alanine, phenylalanine or tryptophan do not have any significant effect on the activity of PEP synthetase.

3. Effect of metal ions on the activity of PEP synthetase

Table VIII shows that Cu²⁺, Ca²⁺ and Fe²⁺ are inhibitors of PEP synthetase. Cu²⁺ at a concentration of 5 mM completely inhibits enzyme activity, 5 mM Ca²⁺ inhibits 70%, whereas 5 mM of Fe²⁺ inhibits 30%. NH₄⁺ or K⁺ at 5 mM have no effect on enzyme activity.

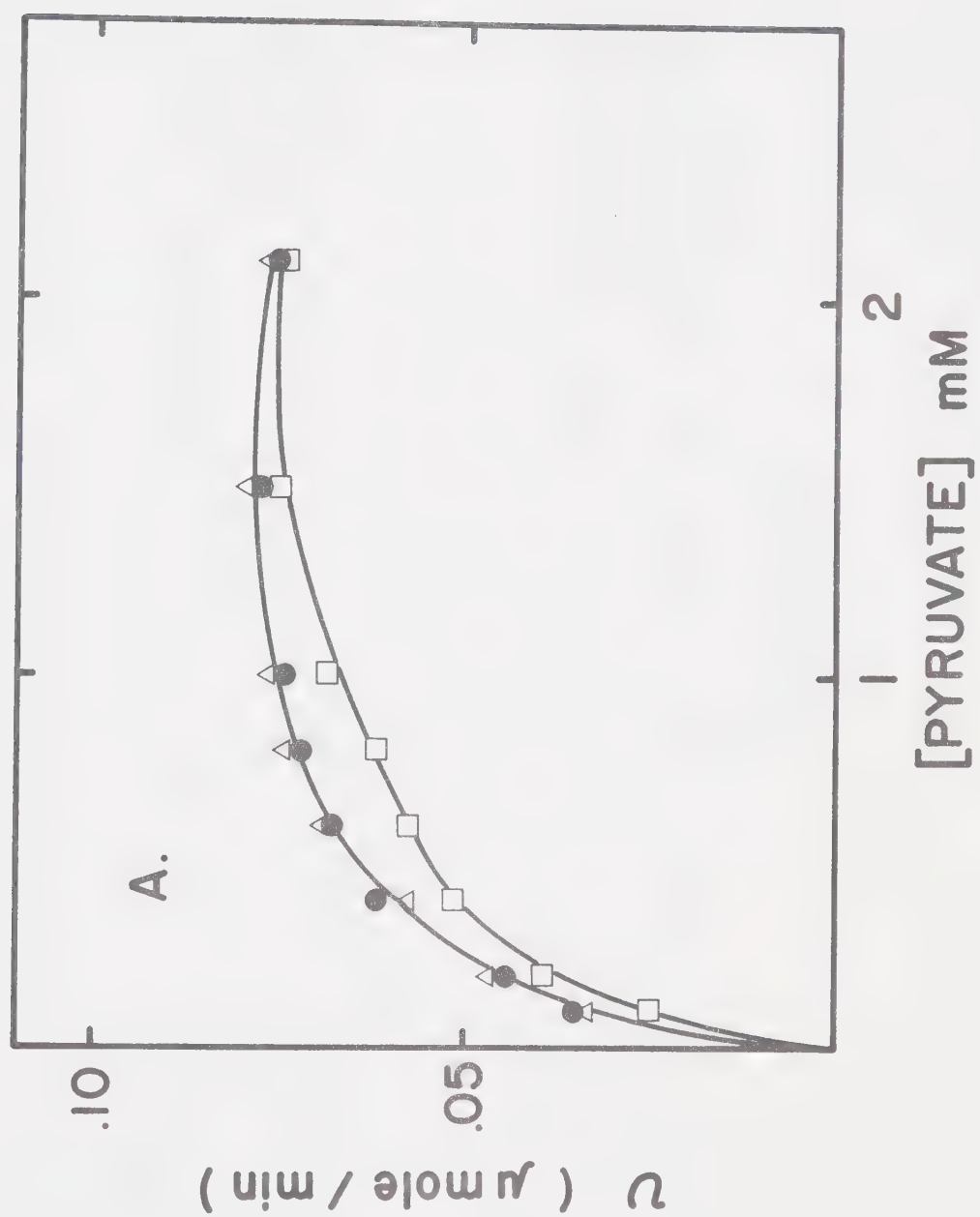
TABLE VI
Apparent K_m of Substrates of PEP Synthetase

Reactant	K_m (mM)
Forward reaction (V_{max} 18 μ mole/min/mg)	
Pyruvate	0.145
ATP	0.435
Reverse reaction (V_{max} 0.83 μ mole/min/mg)	
PEP	0.073
AMP	0.057

Figure 16. Effect of F-1,6-P₂ on the activity of PEP synthetase.

A. Pyruvate is variable substrate. B. ATP is variable substrate.

●—● control, Δ—Δ plus 4mM F-1,6-P₂, □—□ plus 10mM F-1,6-P₂.



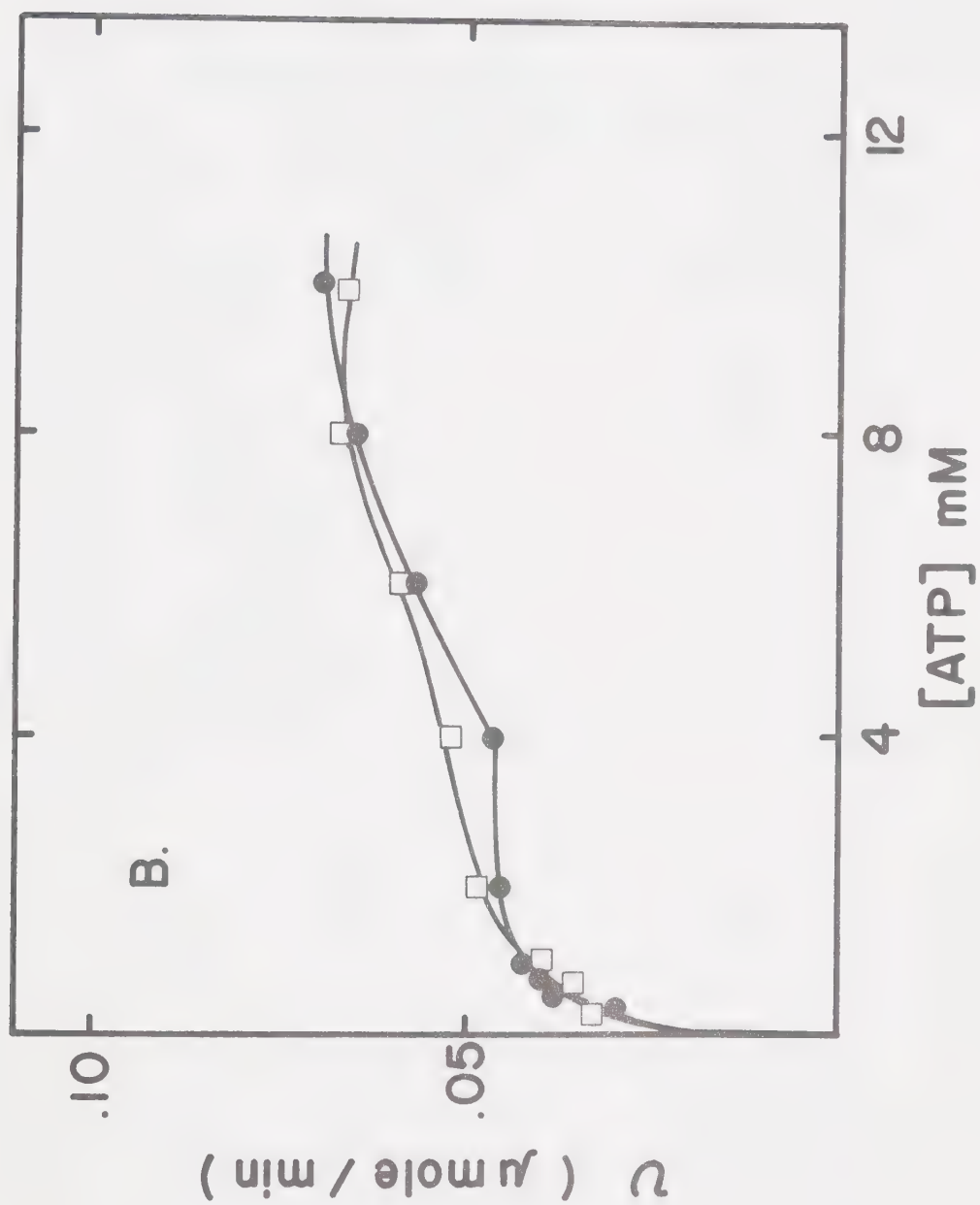


TABLE VII

Effect of Various Metabolites on the Activity
of PEP Synthetase

Metabolites	Relative activity
Control	100
Citrate (10 mM)	95
Alanine (10 mM)	105
Phenylalanine (10 mM)	102
Tryptophan (10 mM)	97
NADH (0.5 mM)	100

TABLE VIII
Effect of Metal Ions on the Enzyme Activity

Metal ions (5 mM)	Relative activity
None	100
Cu ²⁺	0
Ca ²⁺	30
Fe ²⁺	71
NH ₄ ⁺	100
K ⁺	100

4. Effect of oxalate on the activity of PEP synthetase

Oxalate, considered to be an analogue of enol-pyruvate (82, 95), strongly inhibits the reaction catalysed by PEP synthetase. Double reciprocal plots shown in Fig. 17 indicate that oxalate is a competitive inhibitor with respect to pyruvate in the forward reaction, whereas it is noncompetitive with respect to PEP in the reverse reaction (Fig. 18). The plots of $1/v$ against oxalate concentration at various concentrations of pyruvate give an inhibition constant for oxalate of $18 \mu\text{M}$, which is about one order of magnitude lower than the K_m for pyruvate.

D. DISCUSSION

The results obtained from this study agree well with the report by Cooper and Kornberg that the reaction of PEP synthetase favors the direction of PEP formation (46). The maximum velocity of the forward reaction is almost 25 times higher than that of the reverse. Thus under physiological conditions the reaction of PEP synthetase would seem irreversible toward PEP formation. This is opposite to the reaction of pyruvate, phosphate dikinase that has been reported to favor the direction of pyruvate formation (10, 12).

The appearance of the intermediary plateau region of the saturation curve for the phosphorylated substrates has not been noticed by other authors. While this discrepancy may be due to differences in the enzyme preparations, this phenomenon occurs at quite high concentrations of ATP. Most other studies on the enzyme (13, 14) made use of ATP concentrations of only 1 to 2 mM as the saturated concentration. This type of saturation curve has been observed with various enzymes (85 - 89) and subsequently has been related to the finding of half-of-the-

Figure 17. Competitive inhibition by oxalate with respect to pyruvate. The initial rates of forward reaction were measured as described in the text, with pyruvate as variable substrate. Oxalate was present at the concentration indicated.

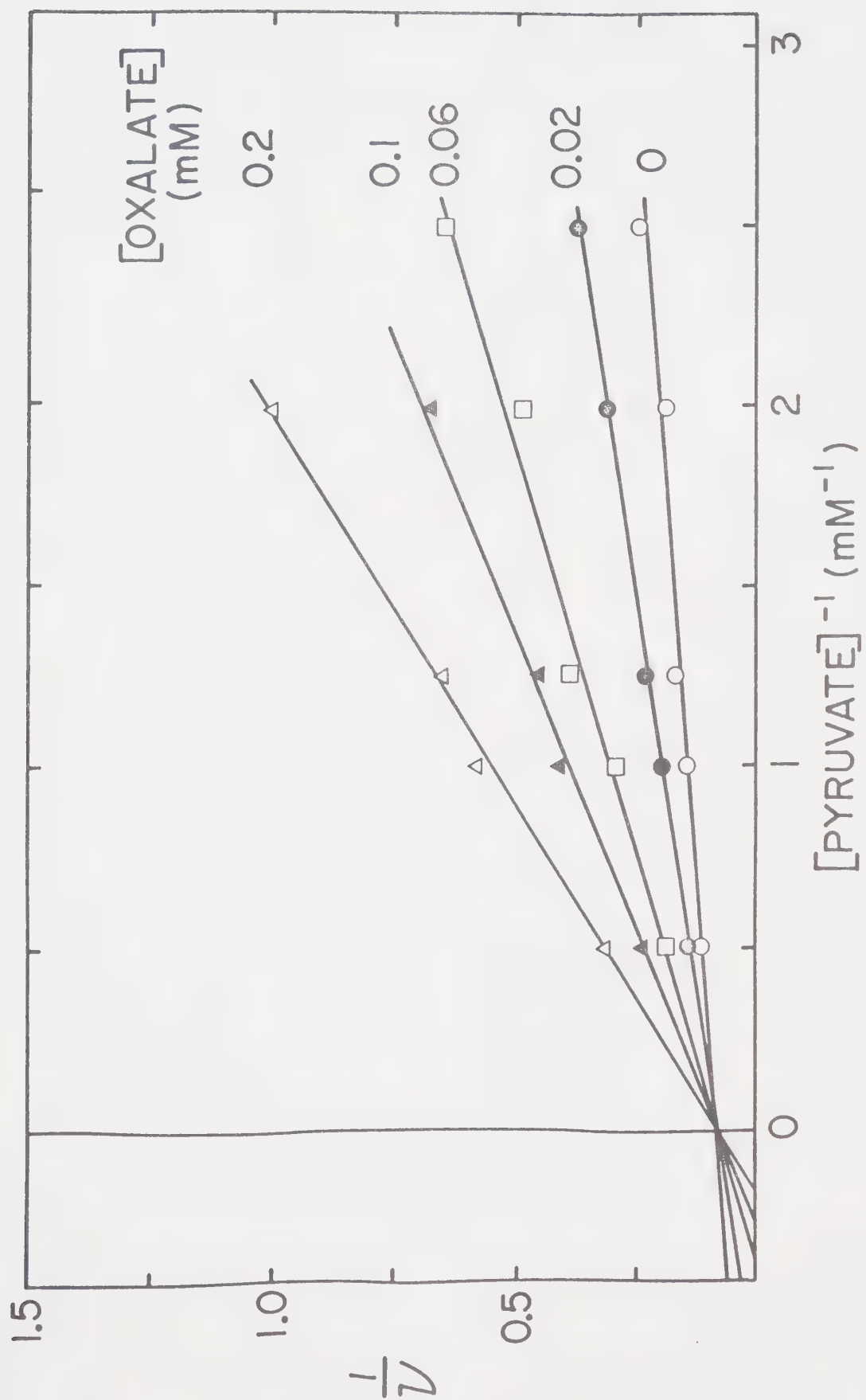
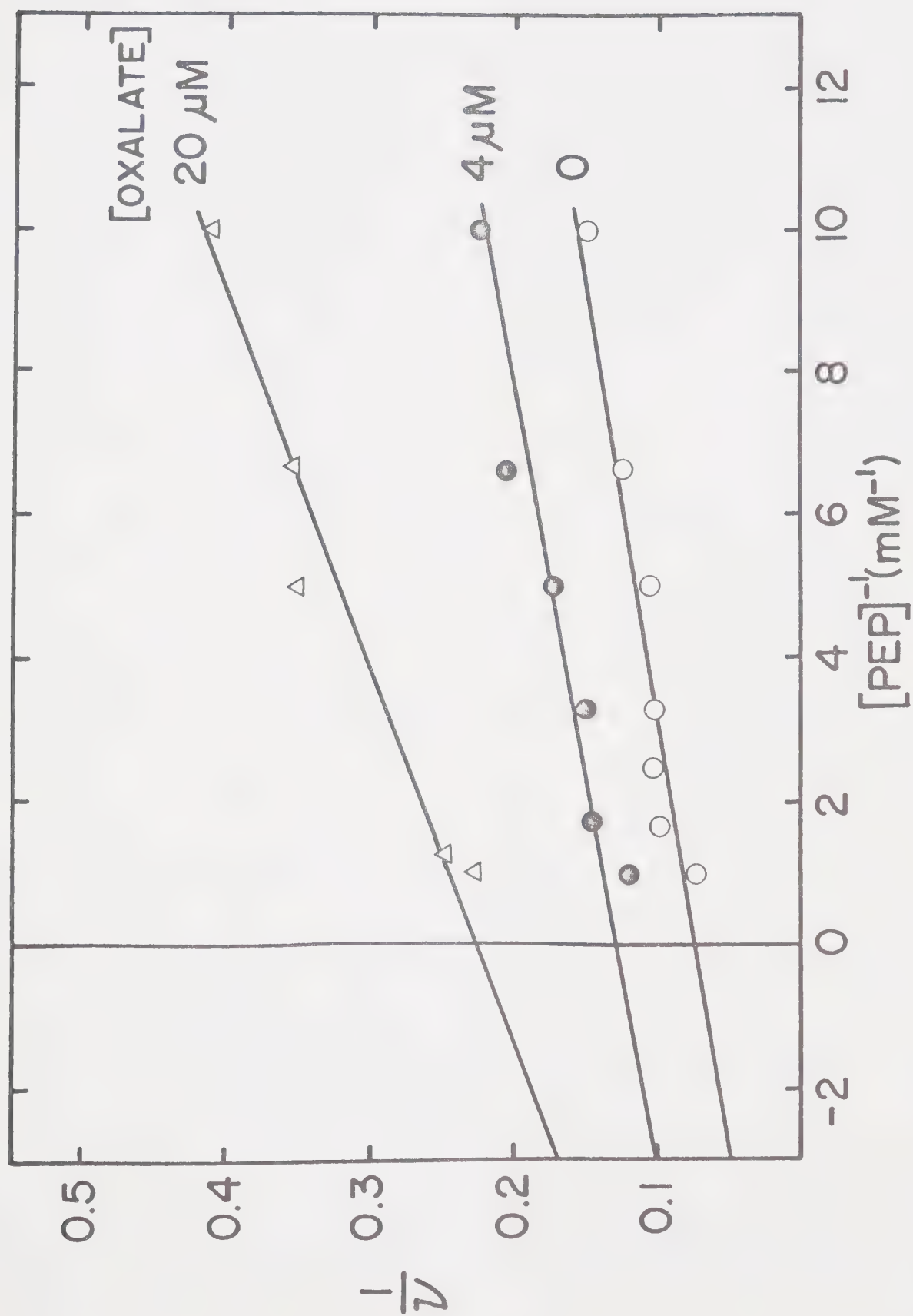


Figure 18. Noncompetitive inhibition by oxalate with respect to PEP. The initial rates of reverse reaction with measured as described in the text, with PEP as variable substrate.



sites reactivity or negative cooperativity (85, 90, 91). In the case of PEP synthetase it has been reported in Chapter IV that despite its dimeric structure, only one phosphoryl group is incorporated per mole of the enzyme. This finding, together with the nature of the saturation curve of ATP, suggests that the enzyme shows negative cooperativity toward phosphorylation by ATP. The phosphorylation of one of the two active sites may cause a conformational change that may prevent the phosphorylation of the second site or may make the binding at the second site so unfavorable or unstable that upon isolation we can detect only one phosphoryl group per mole of enzyme.

The failure to detect any significant effect of various metabolites on the activity of PEP synthetase indicates that the enzyme must be mainly regulated only by end products and by energy charge as previously reported by various authors (14, 46). Product inhibition by AMP (14, 46), together with AMP-activation of pyruvate kinase, may be sufficient to prevent futile cycling. Furthermore, the F-1,6-P₂-activated pyruvate kinase has been reported to be an inducible enzyme which functions during glycolysis (83). During the period that E. coli are grown on a 3-carbon source it is likely that the F-1,6-P₂ activated pyruvate kinase is repressed. Thus, reciprocal induction and repression of PEP synthetase and F-1,6-P₂-activated pyruvate kinase would obviate the necessity for reciprocal allosteric controls.

The inhibitory effects of divalent metal ions on the activity of PEP synthetase are similar to those reported by Evans and Wood (92) for the enzyme pyruvate phosphate dikinase. However, though NH₄⁺ has been reported to activate the reaction of pyruvate phosphate dikinase (92, 93), it has no effect on PEP synthetase. The inhibition by Ca²⁺

is characteristic of Type II metal function, as described by Cohn (94). From magnetic resonance studies of metal activation of enzyme reactions, Cohn (94) has classified the involvement of metal ion on enzyme activity into two groups. For the first group (Type I) the metal atom does not function as a bridge atom between the substrate and the enzyme, but remains bound to the nucleotide only, even in the ternary complex. The enzymes of Type I are activated by Ca^{2+} . In the second group (Type II), the metal is bound to the enzyme and may or may not act as a bridge atom in the ternary complex. The enzymes of this group are inhibited by Ca^{2+} . In fact, it has been reported that the divalent metal ion (Mn^{2+}) forms a complex with PEP synthetase (13). Equilibrium binding studies with electron spin resonance and proton relaxation rate measurements have revealed that Mn^{2+} binds directly to the dephosphoenzyme and the number of binding sites is between three and four per mole of the enzyme (13).

Oxalate was found to strongly inhibit various enzymes that have pyruvate or PEP as substrate or product, and in most cases the K_i for oxalate is much lower than the K_m for pyruvate (92, 95-97). It inhibits rabbit muscle pyruvate kinase competitively with respect to PEP (95) whereas it is competitive with pyruvate for the enzyme pyruvate, phosphate dikinase (82). Our results obtained with PEP synthetase clearly indicate that oxalate competes with the free enol-pyruvate but not with phosphoenolpyruvate. From magnetic resonance studies, Michaels et al. (82) found that oxalate bound so strongly to the Mn(II) -phosphoenzyme of pyruvate, phosphate dikinase that it was clearly acting as a transition state analogue. Since oxalate appears to be acting in a similar fashion with PEP synthetase, this strengthens the argument in favor of

similar catalytic mechanisms for these two enzymes.

CHAPTER VI

CHEMICAL MODIFICATION OF PEP SYNTHETASE

A. INTRODUCTION

Chemical modification of specific amino acid residues within enzymes has provided much information on the role of individual residues in enzyme structure and function. As a result of chemical modification studies, there has recently been an increased awareness of the participation of lysyl, histidyl or arginyl residues, at or near phosphate binding sites of many phosphoryl-transfer enzymes.

In several cases, lysyl residues have been identified by the use of pyridoxal-P as a chemical modifier. Pyridoxal-P appears to act as a site-directed reagent for many enzymes that bind a sugar phosphate (98, 99). Histidyl residues have been implicated in the catalysis of several phosphoryl-transfer enzymes (20, 22, 26, 27).

Until recently, modification of histidyl residues was generally achieved by photooxidation. However, the photooxidation method is not entirely specific for histidyl residues but oxidizes other amino acid residues as well (100). Ethoxyformic anhydride (EFA) has been shown by several authors to be a specific acylating agent of histidyl residues at pH 6.1 (101 - 104).

The recognition of arginyl residues at the active site of enzymes which act upon anionic substrates is largely due to the work of Riordan and co-workers (105 - 108), who have introduced the use of 2,3-butanedione or phenylglyoxal as highly selective reagents for the modification of arginyl residues. Their results have led to the identification of essential arginyl residues in carboxypeptidase A (105), alcohol dehydro-

genase (106), alkaline phosphatase (107), and aldolase (108).

The presence of sulfhydryl residues has also been implicated in active sites of a wide range of phosphoryl-transfer enzymes (109 - 111).

This chapter describes studies of chemical modification of sulfhydryl, histidyl and arginyl residues of PEP synthetase, and the ability of various substrates to protect the enzyme from chemical inactivation.

B. METHODS

1. Modification of sulfhydryl residues

The inactivation kinetics were measured by incubating 3 - 5 nmole of PEP synthetase with the appropriate modifying reagents, in 80 mM potassium phosphate, 1 mM EDTA at the pH indicated in each experiment. An aliquot of modified enzyme was taken for assay at time intervals. Controls of the enzyme incubated under conditions identical to the test experiments, but without modifying reagents, were assayed in companion experiments. Estimation of free sulfhydryl residues was done by using DTNB as described by Ellman (112). All experiments were performed at 25°.

2. Ethoxyformylation of enzyme

Ethoxyformic anhydride solutions were prepared by diluting the reagent into cold absolute alcohol. The anhydride concentration was determined from the increase in absorbance at 230 nm after reaction with 10 mM imidazole pH 7.5, using a molar extinction coefficient for N-ethoxyformyl imidazole of 3000 (113). Ethoxyformylation of the enzyme was initiated by adding the reagent to a solution of enzyme in 0.1 M phosphate, pH 6.8 or 7.5. (The final alcohol concentration in the incubation solution was not more than 2%.) The modification of histidine was followed by the increase in absorbance at 242 nm using the extinction

coefficients of $3.2 \text{ cm}^{-1} \text{ mM}^{-1}$ at pH 6.1 (114), and $2.9 \text{ cm}^{-1} \text{ mM}^{-1}$ at pH 7.5 (104). The reaction was stopped at different time intervals by addition of 10 volumes of 0.1 M Tris-HCl, pH 8.4.

3. Reversal of ethoxyformylation with hydroxylamine

For each experimental point of the time course of reaction with ethoxyformic anhydride, hydroxylamine solution at pH 7.4 was added to final concentration 0.3 M and maintained for 1 hr. Samples were then dialysed overnight to eliminate the reagents. The number of histidyl residues which were still modified were measured by their absorbance at 242 nm, and the residual activity was determined.

4. Modification of arginyl residues

Arginine modification of the enzyme was performed by reacting with phenylglyoxal in 50 mM MOPS buffer pH 7.8.

C. RESULTS AND DISCUSSION

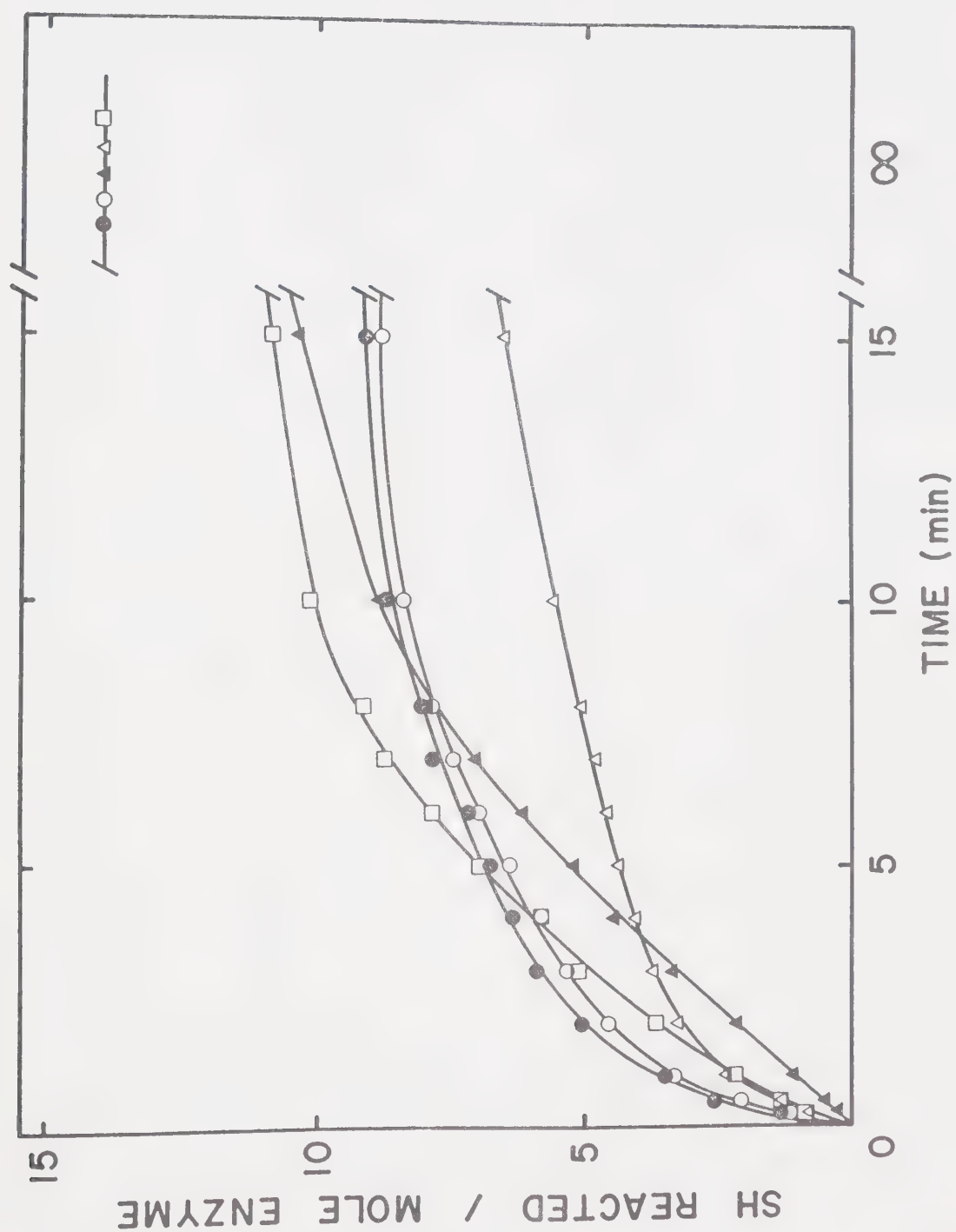
1. Modification of sulfhydryl residues

a. Titration of sulfhydryl residues with DTNB

Fig. 19 shows the rate of titration of enzyme with 0.5 mM DTNB in the absence and presence of various substrates. All of the 14 SH-residues per mole of enzyme (dimer) are accessible to the reagent. They may be arbitrarily classified into 3 groups: Group A consists of 4 residues/mole which react quickly with the reagent, Group B consists of about 4 residues/mole which have intermediate reactivity toward the reagent, and Group C consists of 6 residues/mole which have slow reactivity toward the reagent. Pyruvate alone has no effect on the rate of DTNB titration, but 5 mM pyruvate plus MgCl_2 or 0.33 mM oxalate (an analogue of pyruvate) plus MgCl_2 decreases the rate of titration of both Group A

Figure 19. Titration of PEP synthetase with 0.5 mM DTNB. PEP synthetase (1.7 μ M) was incubated with 0.5 mM DTNB in 0.08 M K-phosphate, 1 mM EDTA, pH 7.5. The number of SH- groups reacted per dimer was determined spectrophotometrically by following the increase in absorbance at 412 nm.

•—•, without substrate; 0—0, plus 5 mM pyruvate; Δ — Δ , plus 5 mM pyruvate and 5 mM MgCl_2 ; $\square$ — $\square$, plus 5 mM ATP + 5 mM MgCl_2 ; $\blacktriangle$ — $\blacktriangle$, plus 5 mM ATP + 0.1 mM oxalate + 5 mM MgCl_2 .



and B. In the presence of 5 mM ATP plus MgCl_2 , the rate of inactivation is dramatically changed, suggesting a conformational change in the enzyme accompanying its phosphorylation. ATP slightly decreases the rate of titration of Group A but greatly enhances the rate of Group B and probably some of the Group C residues. When ATP, MgCl_2 and oxalate are present together, which may produce an approximation of the transition state, the rate of titration of Group A is greatly decreased and that of Group B is enhanced to the same extent as that caused by Mg^{2+} and ATP alone, suggesting that binding of pyruvate or oxalate to the phosphoenzyme may not cause major alteration of the conformation of phosphoenzyme. The rates of titration of these groups are presented in Table IX.

In order to obtain more accuracy of the rate of the fast titratable group, titration of the enzyme with lower concentration of DTNB (0.05 mM) was studied. The correlations between the enzyme activity and number of SH- residues modified with time, in the absence and presence of substrates, are shown in Fig. 20. In the absence of substrate, both the rate of inactivation and the rate of SH- groups modified are biphasic. The first SH- residue is modified quickly resulting in loss of 50% activity, whereas the second, third and fourth residues are modified at the same slower rate. After 3 SH- residues are modified, the enzyme loses about 95% of its activity. The presence of Mg^{2+} plus ATP or Mg^{2+} plus oxalate reduces the rate of DTNB titration and partially protects the enzyme from inactivation. When ATP, Mg^{2+} and oxalate are present together, the rate of DTNB titration is greatly decreased, but the activity is not completely restored after the enzyme is titrated with DTNB for a long period. When the residual activity is plotted against the number of SH- groups modified (Fig. 21), in all cases loss of 1 - 1.5

TABLE IX

Rate of Titration of SH- Groups by 0.5 mM DTNB

Experimental conditions	Rate of SH- titration (min^{-1})		
	Group A	Group B	Group C
Without substrate	0.275	0.075	0.018
Plus pyruvate and MgCl_2	0.223	0.036	0.025
Plus ATP and MgCl_2	0.204	0.113	0.039
Plus ATP, oxalate and MgCl_2	0.126	0.116	0.038

Figure 20. Inactivation of PEP synthetase by DTNB. Enzyme ($8.3 \mu\text{M}$) was incubated with 0.05 mM DTNB, and the number of SH- groups modified was determined (data represented in the bottom panel). Aliquots were also taken at indicated time to determine the residual activity (data represented in the top panel).

●—●, control; Δ — Δ , plus 0.1 mM oxalate + 5 mM MgCl_2 ;
O—O, plus 0.67 mM ATP + 5 mM MgCl_2 ; $\blacktriangle$ — $\blacktriangle$, plus 0.67 mM ATP + 0.1 mM oxalate + 5 mM MgCl_2 .

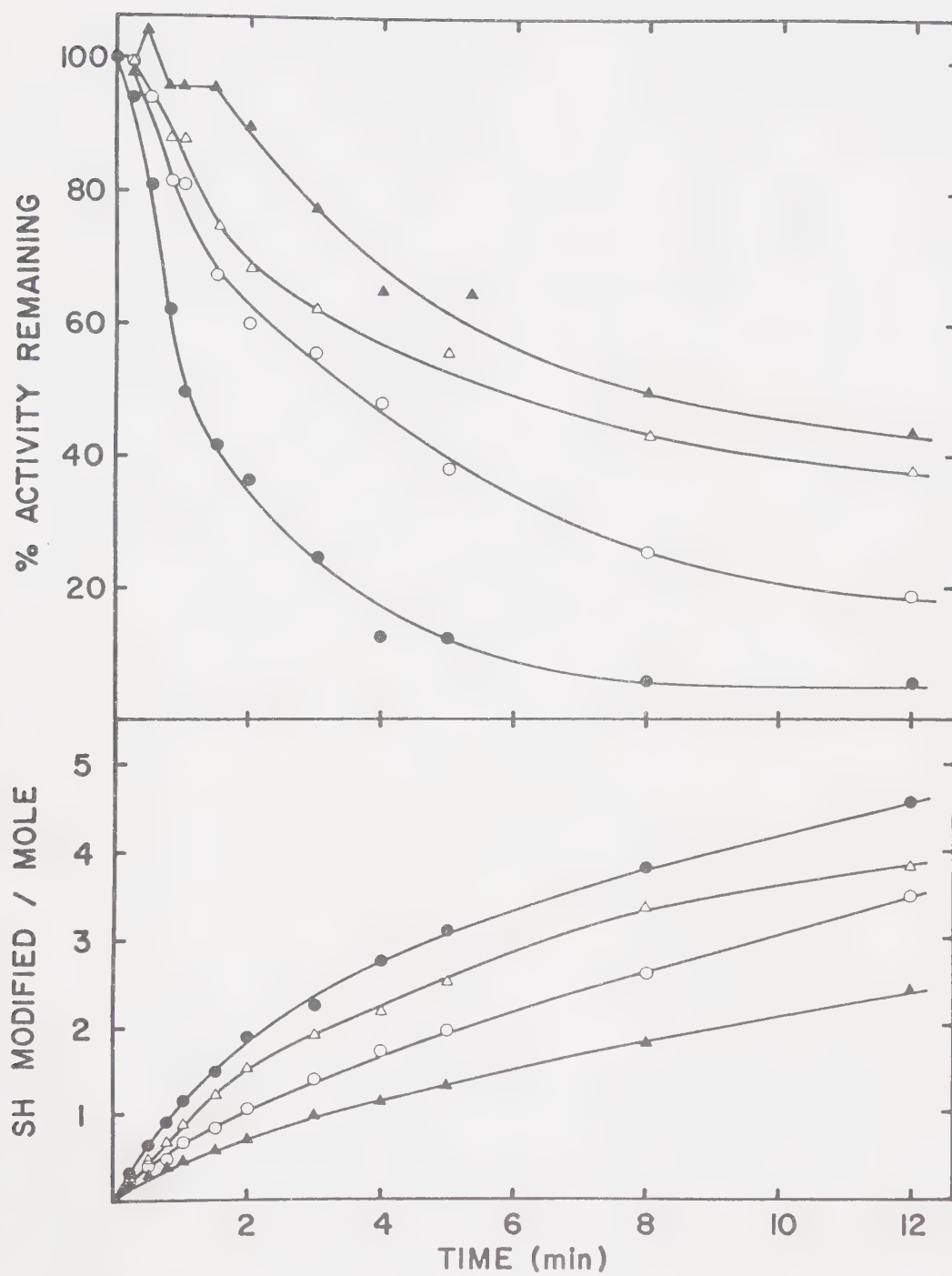
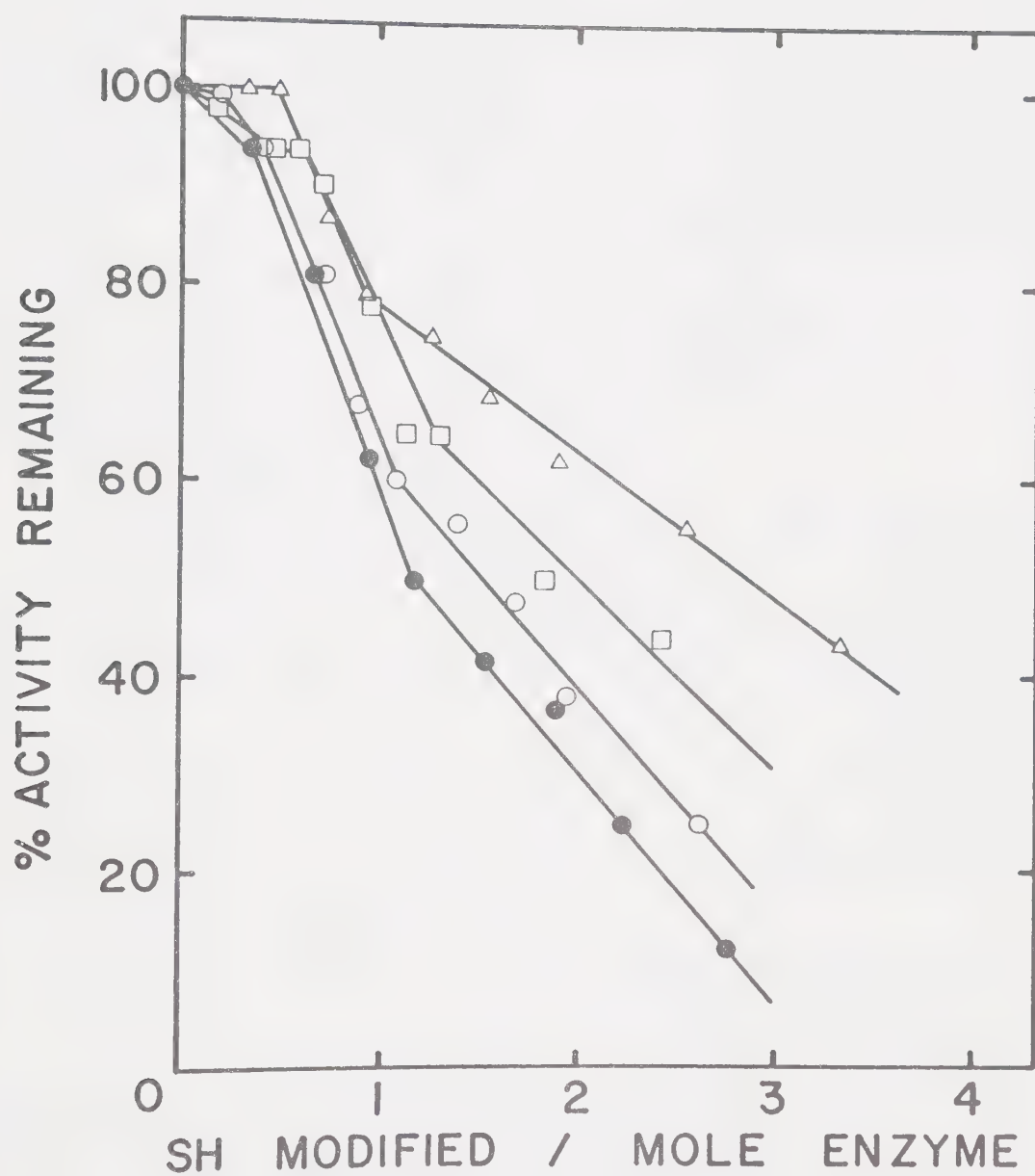


Figure 21. Correlation of SH modification with inactivation of PEP synthetase by DTNB. The system was as described in Fig. 20.



mole of SH- group corresponds to loss of 50% enzyme activity.

The reaction of the enzyme with DTNB seems complex. The multiphasic reaction of the enzyme with the reagent suggests a slow unwinding or rearrangement of the protein structure which occurs differently in the presence and absence of substrates. This, in turn, may result in intra- or interchain disulfide bond formation. Thus, attempts to correlate the stoichiometry of the reactive SH- groups with the catalytic activity may be pointless since it is not possible to distinguish the original essential thiol groups from those which were originally buried and became exposed as a result of conformational rearrangement after being oxidized by DTNB.

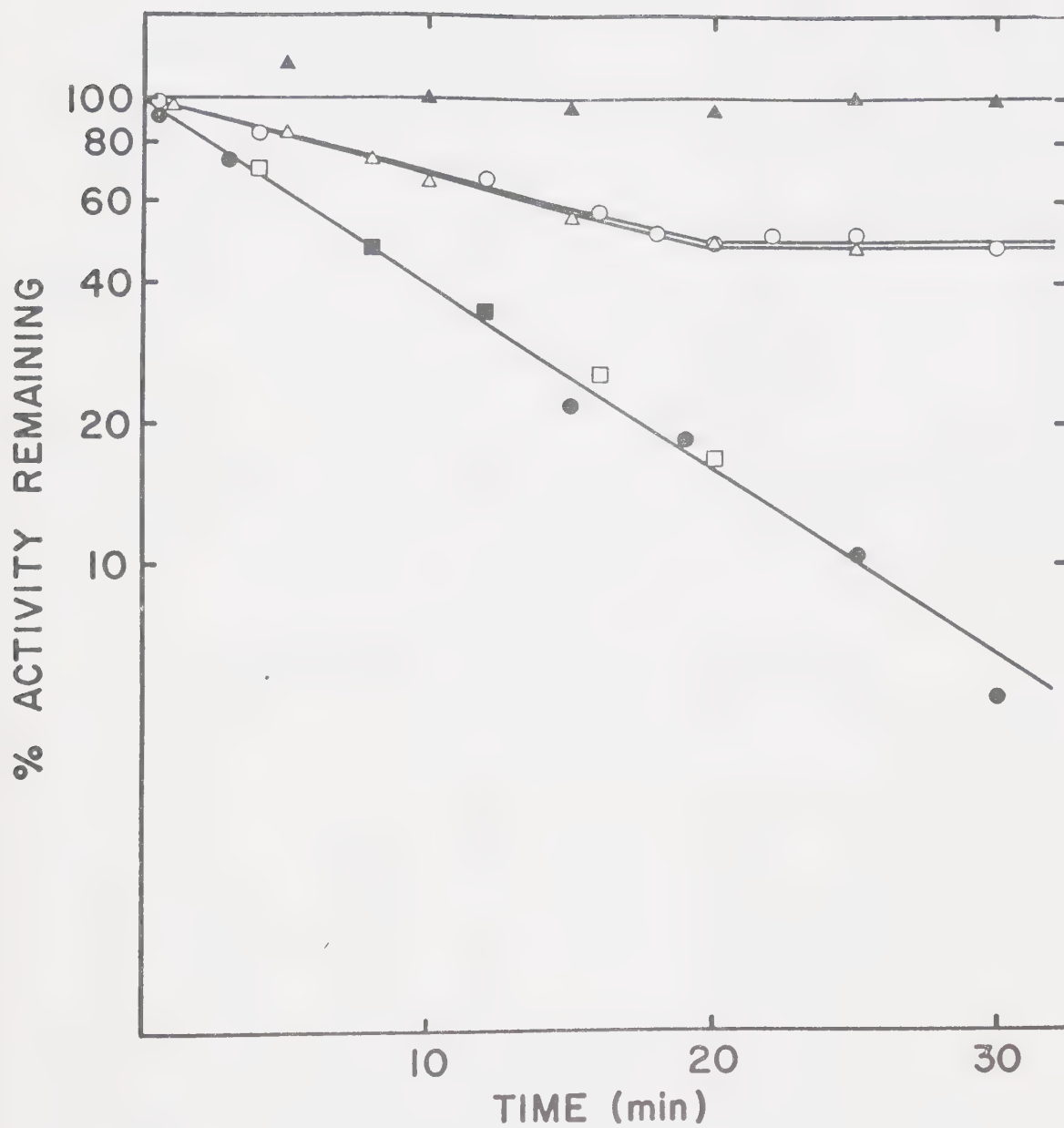
b. Reaction with NEM

PEP synthetase is strongly inhibited by NEM in 80 mM phosphate - 1 mM EDTA, pH 7.0. Fig. 22 shows that about 95% of enzyme activity is lost after 30 min incubation of the enzyme with 0.167 mM NEM. Either ATP plus Mg^{2+} or oxalate plus Mg^{2+} reduces the rate of inactivation equally and about 50% of activity is retained. Significantly, when both ATP and oxalate are present together, they completely protect the enzyme against inactivation by NEM.

In interpreting the protection by ATP, the question may be raised as to whether the protective effect is contributed by formation of phosphoenzyme or by AMP which is present in equivalent amounts. The effect of AMP plus Mg^{2+} on NEM inactivation was also studied, and the results show that AMP does not protect the enzyme against inactivation by NEM at all. Thus it is clear that the protection by ATP is due to phosphoenzyme formation.

One might also ask whether only one or both of the two partial

Figure 22. Inactivation of PEP synthetase by NEM and protection by ATP and oxalate. The enzyme was incubated with 0.167 mM NEM in 0.08 M K.phosphate, 1 mM EDTA, pH 7.0, in the absence and presence of substrates. ●—●, control; ○—○, plus 0.67 mM ATP + 5 mM MgCl_2 ; Δ—Δ, plus 0.1 mM oxalate + 5 mM MgCl_2 ; □—□, plus 0.67 mM AMP + 5 mM MgCl_2 ; ▲—▲, plus 0.67 mM ATP + 0.1 mM oxalate + 5 mM MgCl_2 .

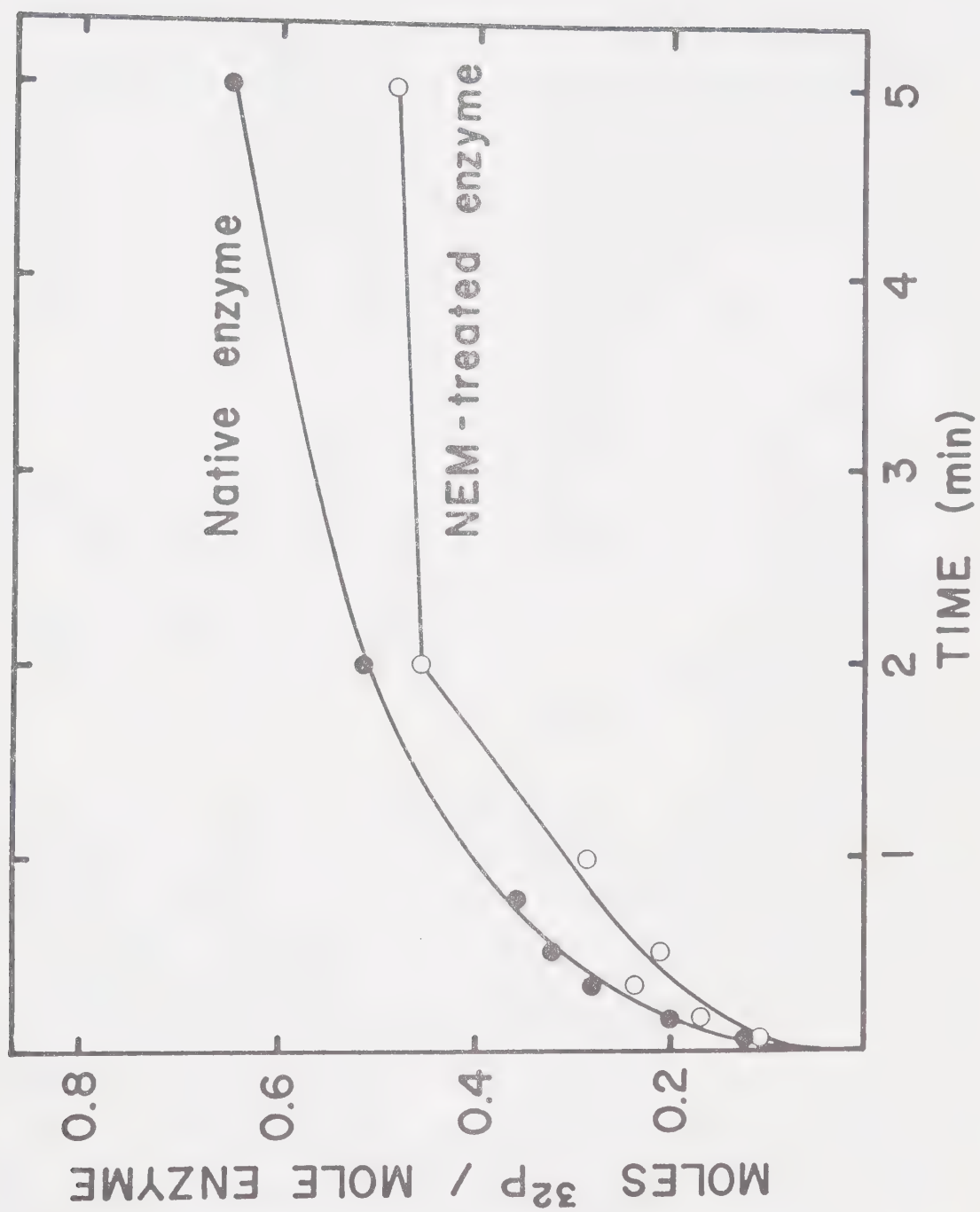


reactions is affected by sulfhydryl modification. To test this, the enzyme was incubated with 1 mM NEM in the absence of substrates for 15 min, which resulted in complete loss of detectable overall catalytic activity. After removal of the excess reagent by passing through a Sephadex column, the NEM-treated enzyme was checked for its ability to undergo phosphorylation using ATP- β - ^{32}P . Fig. 23 shows that indeed the NEM-treated enzyme is phosphorylated, albeit at a slightly slower rate than the native enzyme. From this, we draw the conclusion that the reactive SH- residues may be necessary only for the second partial reaction, i.e. binding of pyruvate to E-P, since the presence of E-P plus oxalate completely protects the activity. The protective effect of ATP may be due to the suspected conformational change caused by phosphoenzyme formation that may result in shielding of the reactive SH- residues from reaction with NEM.

c. Incorporation of ^{14}C -NEM into PEP synthetase

In order to determine the stoichiometry of alkylation, modification of the enzyme by ^{14}C -NEM was also studied, both in the presence and absence of substrates. Table X shows that when the enzyme is incubated with 0.167 mM NEM for 30 min, resulting in loss of 95% of its activity, about 2 moles of ^{14}C -NEM are incorporated per mole of enzyme. In the presence of ATP plus Mg^{2+} the NEM incorporation is only slightly less than in its absence although half of the activity is retained. The explanation for this could be that while ATP causes a conformational change that shields the reactive SH- residues, at the same time it may expose other SH- residues to the reagent. The presence of Mg^{2+} plus oxalate alone protects only 0.5 mole of SH- residue per mole of enzyme and 50% of activity is retained. However, when both ATP and oxalate are

Figure 23. Phosphorylation of NEM-treated enzyme. 0.16 nmole of native or NEM-treated enzyme was incubated with 0.085 mM ATP- β - ^{32}P + 1 mM MgCl_2 . The reaction was stopped at indicated time by means of perchloric acid precipitation, and the radioactivity incorporated was determined after filtration on millipore membrane.



present, giving complete protection against inactivation, only one SH-residue/mole is protected.

Back-titration of the SH- residues with ^{14}C -NEM was also studied. This was done by first incubating the enzyme with unlabeled NEM in the absence and presence of ATP, Mg^{2+} and oxalate under the same conditions described in Table X (experiment A). After 30 min of incubation, the reaction was stopped by adding 2 mM β -mercaptoethanol and the reaction mixture was immediately passed through a Sephadex column. The unlabeled NEM-treated enzyme was then incubated with 0.167 mM ^{14}C -NEM for another 30 min and the incorporation of radioactivity into the treated enzyme was determined. Results shown in Table X (experiment B) indicate that for the enzyme previously treated with unlabeled NEM in the absence of substrate, 1 mole of SH- residue is incorporated further, whereas the enzyme previously treated in the presence of ATP plus oxalate incorporates 2 moles of ^{14}C -NEM. Again, this difference indicates that one SH-residue was protected during the course of modification in the presence of ATP plus oxalate. The contrary seems to prevail for the results obtained from modification in the absence and presence of substrates. However, there is the possibility that 2 SH- residues are equally reactive toward modification by NEM, but only one of these is important for enzyme activity and only this particular residue is protected by ATP plus oxalate. It has been reported earlier that PEP synthetase shows the phenomenon of half-of-the-sites reactivity toward phosphorylation: only one mole of ^{32}P is incorporated per enzyme dimer (Chapter IV). The finding that only one mole of SH- residue per mole of enzyme (i.e. per dimer) is necessary for enzyme activity lends additional support to this concept.

TABLE X
Incorporation of ^{14}C -NEM into PEP Synthetase

Conditions	Mole ^{14}C NEM incorporated/ mole enzyme
Experiment A:	
Control	1.9
ATP + Mg^{2+} added	1.7
Oxalate + Mg^{2+} added	1.4
ATP + oxalate + Mg^{2+} added	1.08
Experiment B:	
Control (after treatment with unlabelled NEM without substrates)	0.9
After treatment with unlabelled NEM in the presence of ATP + oxalate	1.96

Experiment A:

2 nmole of PEP synthetase were incubated with 0.167 mM ^{14}C -NEM in 0.08 M phosphate, 1 mM EDTA pH 7.0, at 25°; in the absence of substrates and in the presence of 0.67 mM ATP + 5 mM MgCl_2 , or 0.33 mM oxalate + 5 mM MgCl_2 , or the combination of ATP + oxalate + Mg^{2+} . The reaction was stopped after 30 min by addition of 2 mM mercaptoethanol. The protein was then precipitated with equal volume of 0.4 M perchloric acid in cold, filtered on millipore filter (5 μ), washed several times with cold 0.2 N perchloric acid, and counted for radioactivity.

Experiment B:

The enzyme was treated with unlabelled NEM in the absence or presence of ATP + oxalate + Mg^{2+} under the same conditions as in Experiment A. After 30 min of incubation, the reaction was stopped by adding 2 mM mercaptoethanol. The protein was passed through Sephadex column to remove excess reagents. The treated enzyme was then incubated with 0.167 mM ^{14}C -NEM for 30 min. The radioactivity incorporated into the protein was determined as described above.

d. Inhibition by iodoacetamide

PEP synthetase is also inhibited by iodoacetamide, though at higher concentrations of the reagent than those of DTNB and NEM. The pH-dependence of the inactivation rate is shown in Fig. 24. The effects of ATP or oxalate, or both, on protection of enzyme against inactivation by 10 mM reagent were studied at pH 7.0, 7.5 and 8.0. The results are shown in Fig. 25, 26, and 27, respectively. At pH 7.0 (Fig. 25), ATP or oxalate equally reduce the rate of inactivation by iodoacetamide. When both ATP and oxalate are present, the rate of inactivation is greatly decreased, but the activity is not completely retained as in the case of NEM modification. At pH 7.5 (Fig. 26), the protective effect of ATP is less pronounced than that of oxalate but the presence of both reactants still protects the enzyme activity more effectively than the presence of oxalate alone. However, at pH 8.0, ATP does not protect the enzyme against inactivation by iodoacetamide at all, whereas oxalate still has a protective effect. When present together with oxalate at this pH, ATP does not contribute any additional protection beyond that provided by oxalate alone (Fig. 27).

The ability of oxalate to protect the enzyme against inactivation by iodoacetamide at all pH's studied supports the idea that SH- residues are essential for pyruvate binding. While less can be said about the effect of ATP on SH- modification, perhaps different conformational states of the phosphoenzyme at different pH's may have altered reactivity toward these reagents. Indeed, the results from the CD study of the phosphoenzyme at pH 7.0 and pH 8.0, as reported in Chapter III, indicate a pronounced conformational change accompanying phosphorylation at pH 8.0. Furthermore, it has been found in the case of DTNB modification

Figure 24. Effect of pH on rate of inactivation by iodoacetamide. The enzyme was incubated with 10 mM iodoacetamide in 0.08 M K.phosphate, 1 mM EDTA at indicated pH. Rates of inactivation were determined from linear semi-log plots of residual activity versus time.

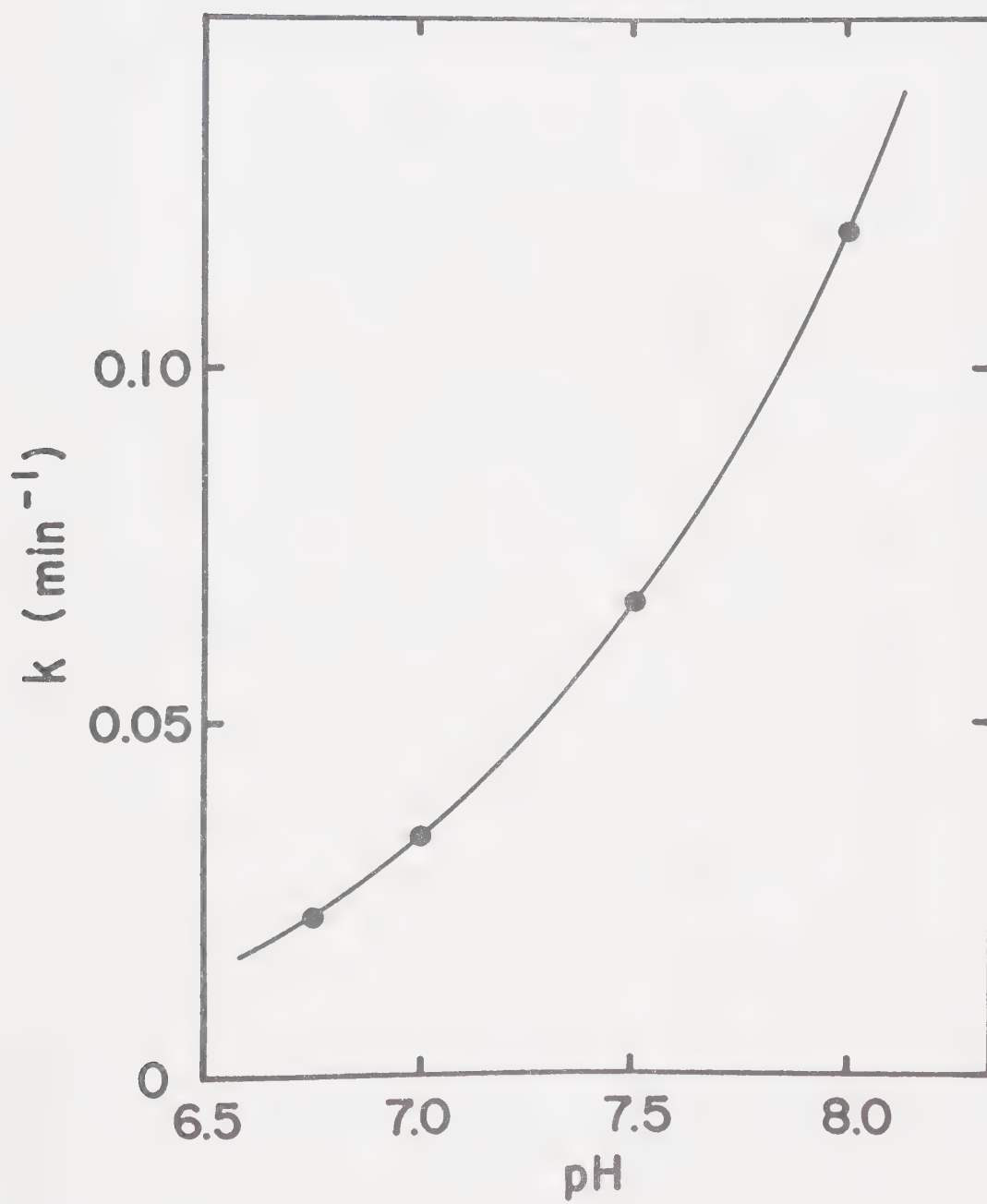


Figure 25. Inactivation of PEP synthetase by iodoacetamide at pH 7.0.

The enzyme was incubated with 10 mM iodoacetamide at pH 7.0 in the absence and presence of substrates: ●—●, control; ○—○, plus 0.67 mM ATP + 5 mM MgCl_2 ; Δ — Δ , plus 0.33 mM oxalate + 5 mM MgCl_2 ; □—□, plus 0.67 mM ATP + 0.33 mM oxalate + 5 mM MgCl_2 .

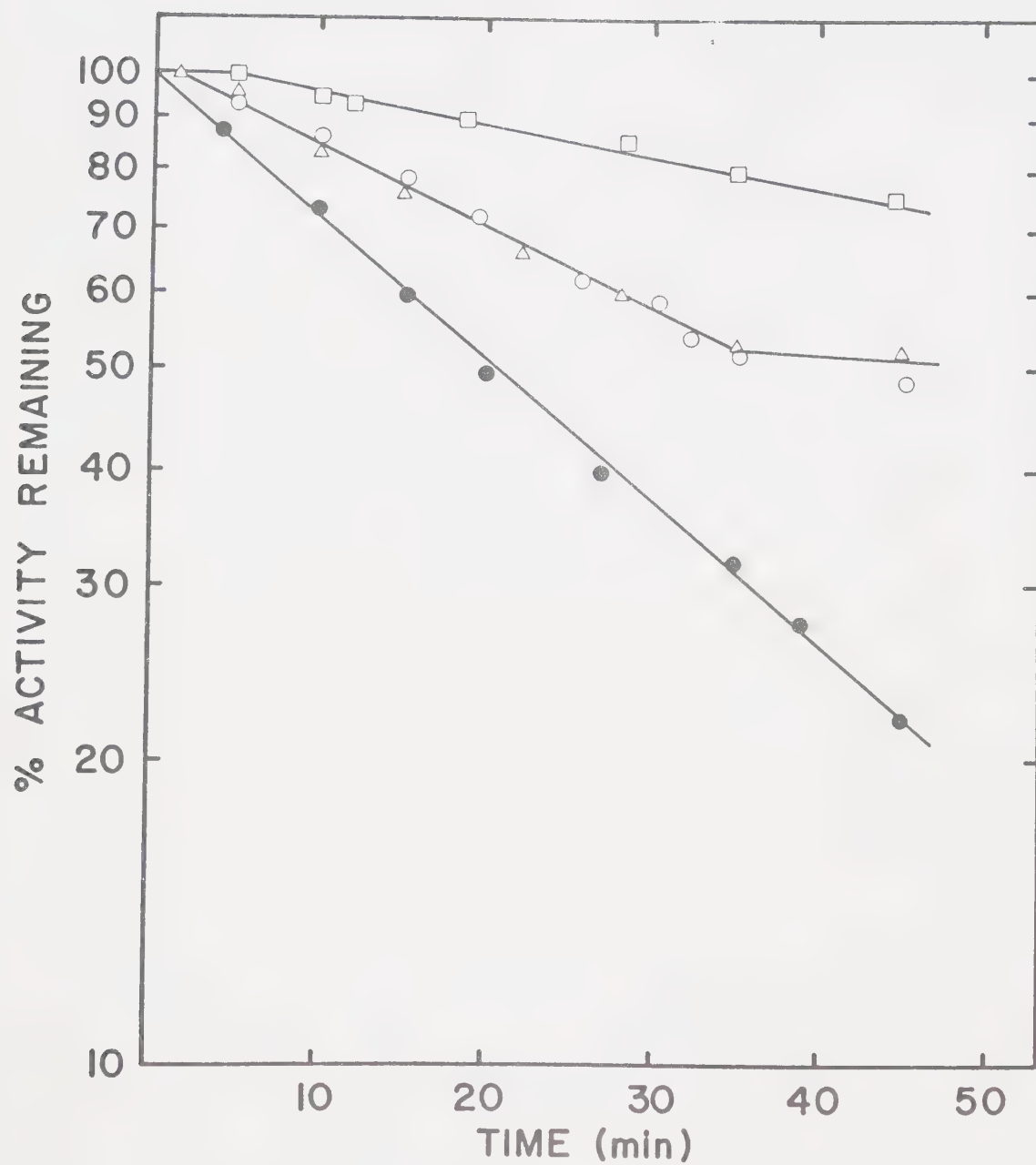


Figure 26. Inactivation of PEP synthetase by 10 mM iodoacetamide at pH 7.5. O—O, control; O—O, plus 0.67 mM ATP + 5 mM MgCl_2 ; Δ — Δ , plus, 0.33 mM oxalate + 5 mM MgCl_2 ; $\square$ — $\square$, plus 0.67 mM ATP + 0.33 mM oxalate + 5 mM MgCl_2 .

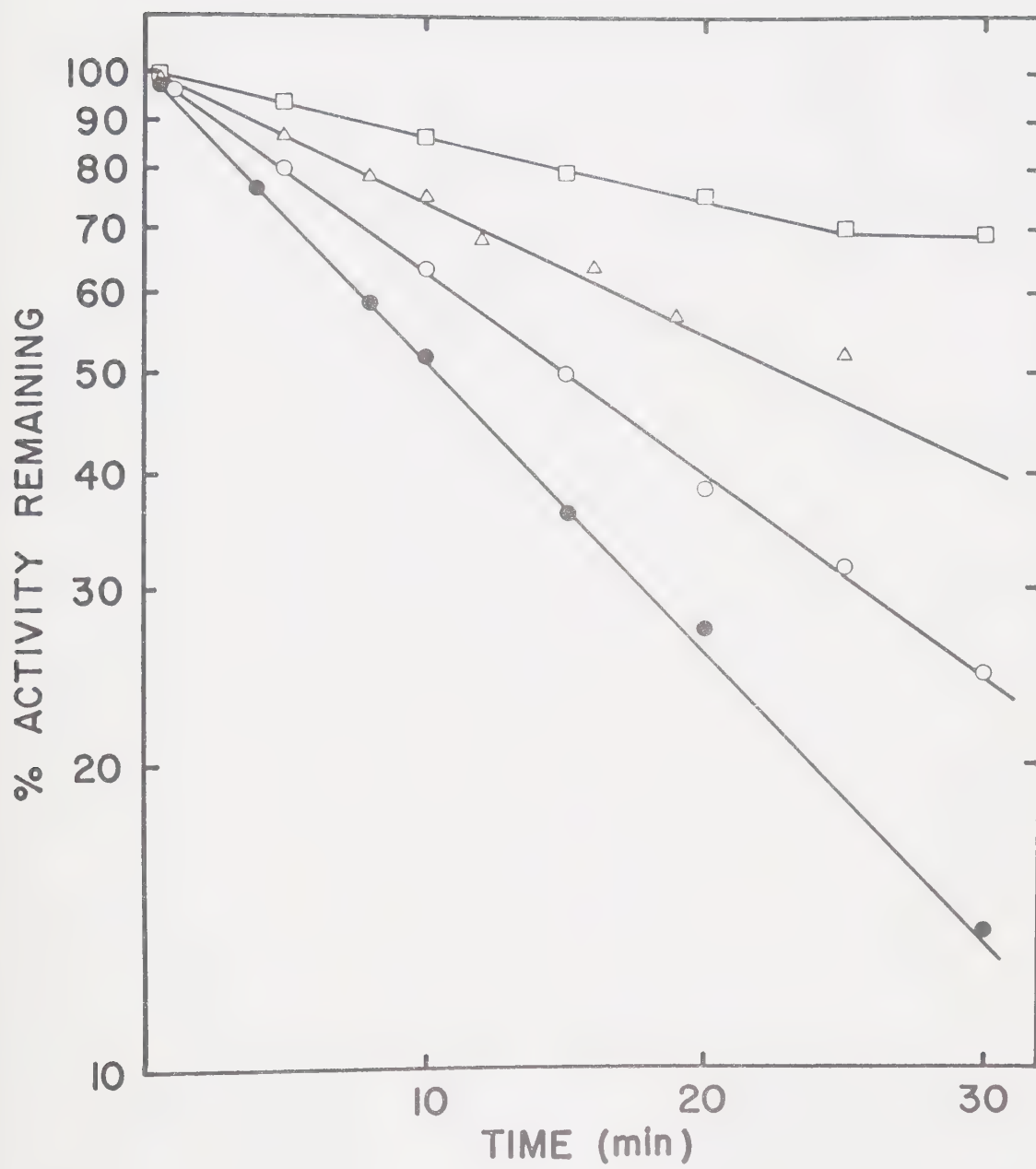
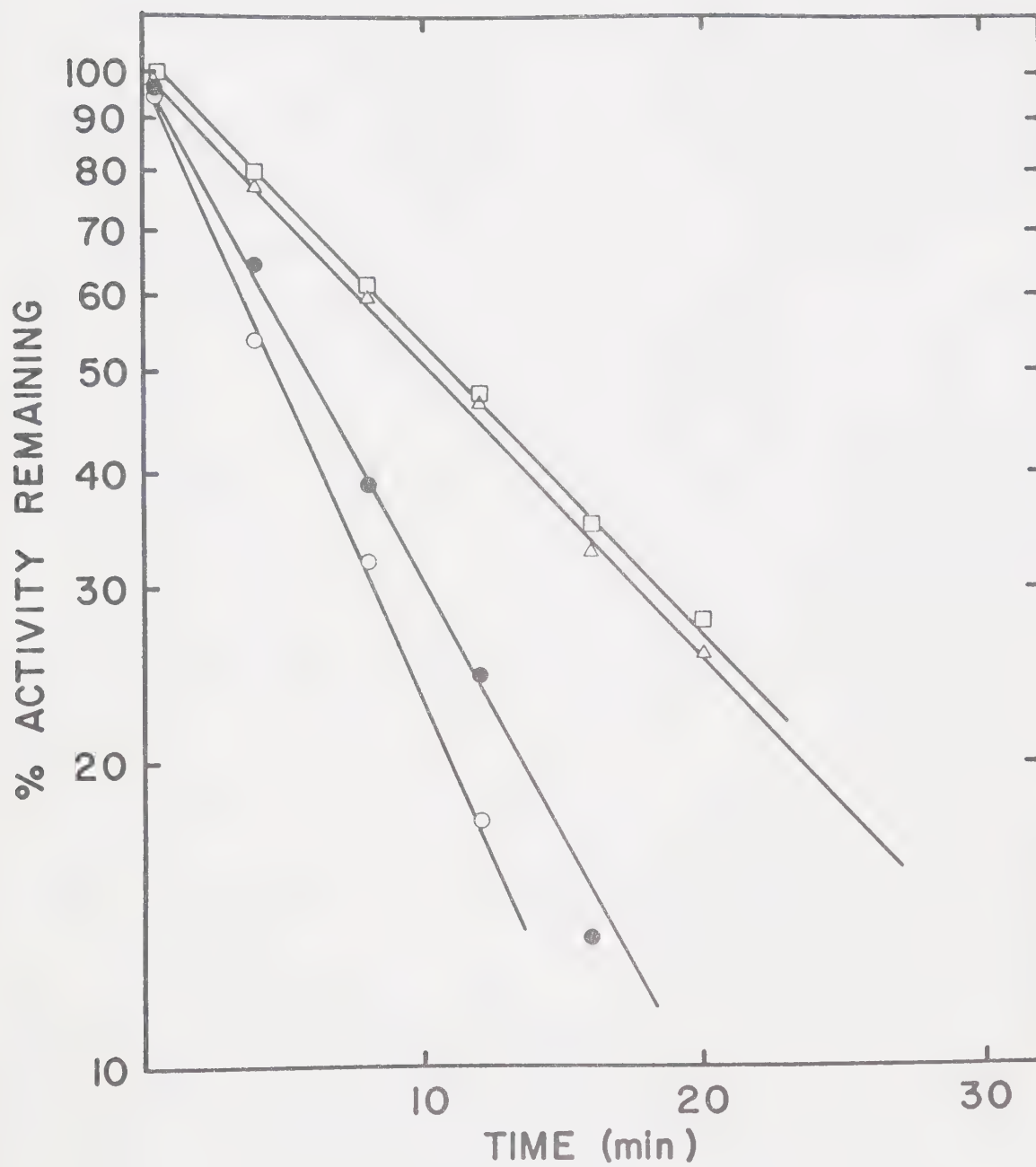


Figure 27. Inactivation of PEP synthetase by 10 mM iodoacetamide at pH 8.0. ●—●, control; ○—○, plus 0.67 mM ATP + 5 mM MgCl_2 ; Δ—Δ, plus 0.33 mM oxalate + 5 mM MgCl_2 ; □—□, plus 0.67 mM ATP + 0.33 mM oxalate + 5 mM MgCl_2 .



that while ATP protects the essential SH- residues, its presence enhances the rate of modification of other SH- residues at the same time; this may also be true in the case of modification by iodoacetamide. Especially at pH 8.0 when most of the SH- residues are likely more reactive (usual pK for cysteine-SH is near 8.3), any protective effect of ATP may be abolished by modification of other SH- residues that may not be essential for substrate binding or catalytic activity, but may be necessary for maintaining the enzyme structure. At lower pH (i.e., pH 7.0), the reagent may selectively modify the residues that have unusually low pK, perhaps the ones situated near the enzyme's active site.

Results obtained from various sulfhydryl inhibitors, as reported in this chapter, strongly suggest the involvement of SH- residues in substrate binding. This is in contrast to the preliminary results reported by Berman and Cohn (13) to the effect that iodoacetate or p-mercuribenzoate inhibited the enzyme activity, but that the rate of inactivation was not affected by Mg^{2+} , pyruvate or ATP either singly or in various combinations. From our studies, the protective effect of oxalate, the analogue of enol-pyruvate, is consistent for reagents and pH's used. Though the involvement of SH- residues in pyruvate binding is quite likely, the possibility that they may be involved merely via maintenance of protein structure cannot be ruled out. For example, studies on reactive thiol groups of rabbit muscle creatine kinase have led to the conclusion that the reactive thiol groups are not concerned with the binding of substrates, although nucleotides or guanidine substrates partially protect the enzyme against inactivation by iodoacetate (115) and the presence of both substrates completely protects the enzyme against inactivation by iodoacetamide (116). The demonstration that

metal complexes of ADP and ATP bind well to the carboxymethylated or oxidized enzyme (115, 117), and the finding that guanidino substrates are also bound to creatine kinase which has been inactivated by reaction with a nitroxide spin-labeled derivative of iodoacetamide (117) led to the conclusion that the essential reactive SH- residues cannot be directly involved in the binding of either substrate. Therefore, it has been proposed that the reactivity of the essential SH- residues is closely linked to the conformational changes that occur when both substrates are bound to the enzyme.

2. Modification of histidyl residues by ethoxyformic anhydride

a. Rate of enzyme inactivation and stoichiometry of modification

The rates of enzyme inactivation and histidine modification by ethoxyformic anhydride were studied at pH 6.1 and pH 7.5. At pH 7.5, both the rate of histidine modification and of inactivation are faster than at pH 6.1 (Fig. 28). However, the extent of inactivation correlates with the number of histidine residues modified at either pH. The first 50% of the enzyme activity is lost at a very fast rate and corresponds to 2 moles of histidyl residues modified (Fig. 29). Complete loss of enzyme activity is observed only after more than eight histidine residues are modified.

b. Effect of substrates on the rate of histidine modification

Since we had already identified phosphohistidine as the phosphorylated residue in the phosphoenzyme (Chapter IV), our main goal was to determine whether PEP or ATP could protect the enzyme from inactivation by ethoxyformic anhydride. Fig. 30 shows that both PEP and ATP protect the enzyme activity from early inactivation, though not completely. ATP is more effective than PEP in protecting the enzyme activity in

Figure 28. Rate of inactivation and modification of histidyl residues by ethoxyformic anhydride at pH 6.1 (▲—▲) and 7.5 (●—●). The enzyme (3.6 μ M) was incubated with ethoxyformic anhydride (0.42 mM). The number of histidyl residues modified was measured from the increase in absorbance at 242 nm. Aliquots were taken at indicated times to determine residual activity.

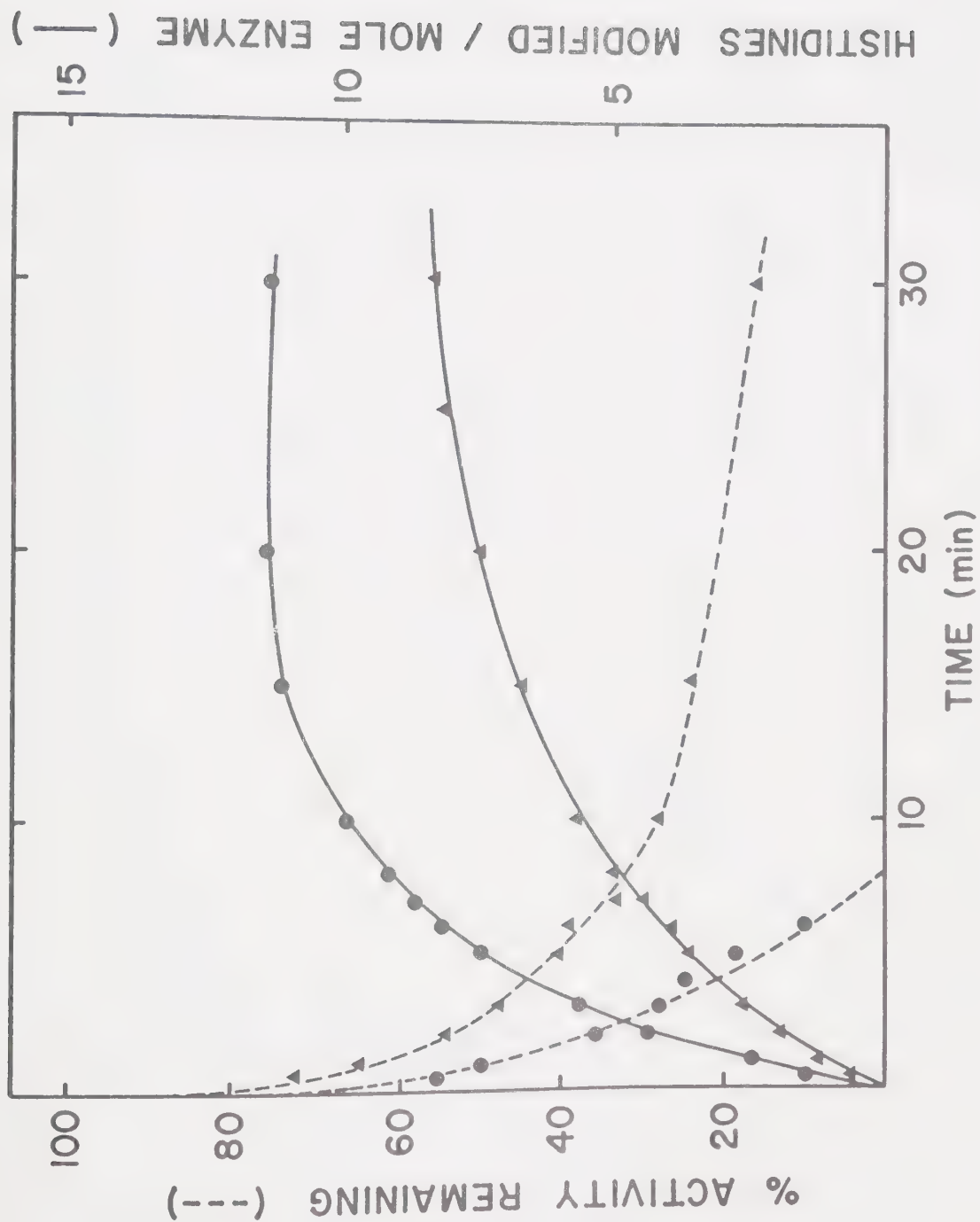


Figure 29. Stoichiometry of histidine modification and rate of inactivation by ethoxyformic anhydride. Data obtained from Fig. 28.

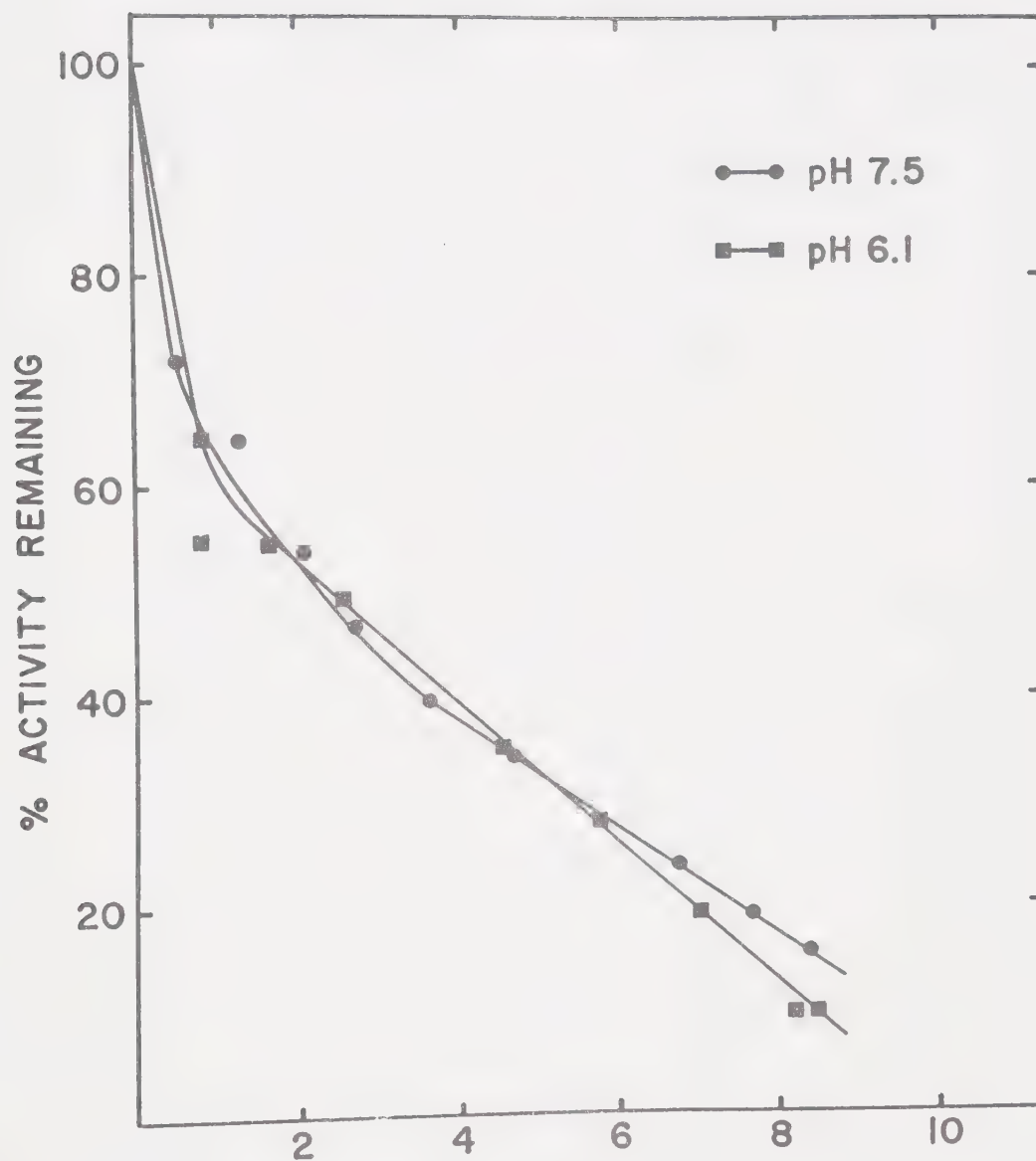
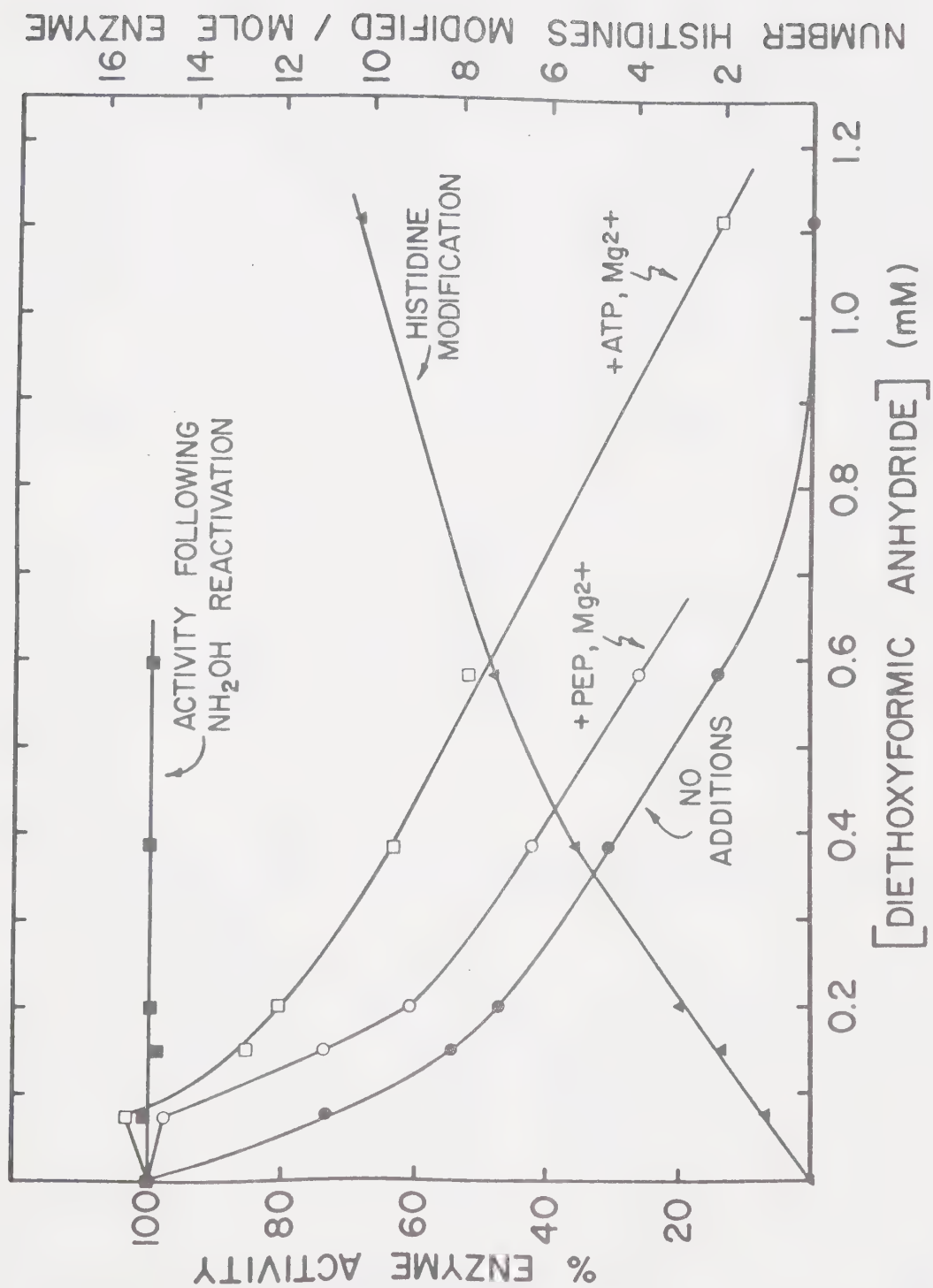


Figure 30. Effect of substrates on inactivation by ethoxyformic anhydride. The enzyme was incubated with ethoxyformic anhydride at pH 6.1 in the absence and presence of substrates as indicated. Also shown is the reversal of inactivation by hydroxylamine.



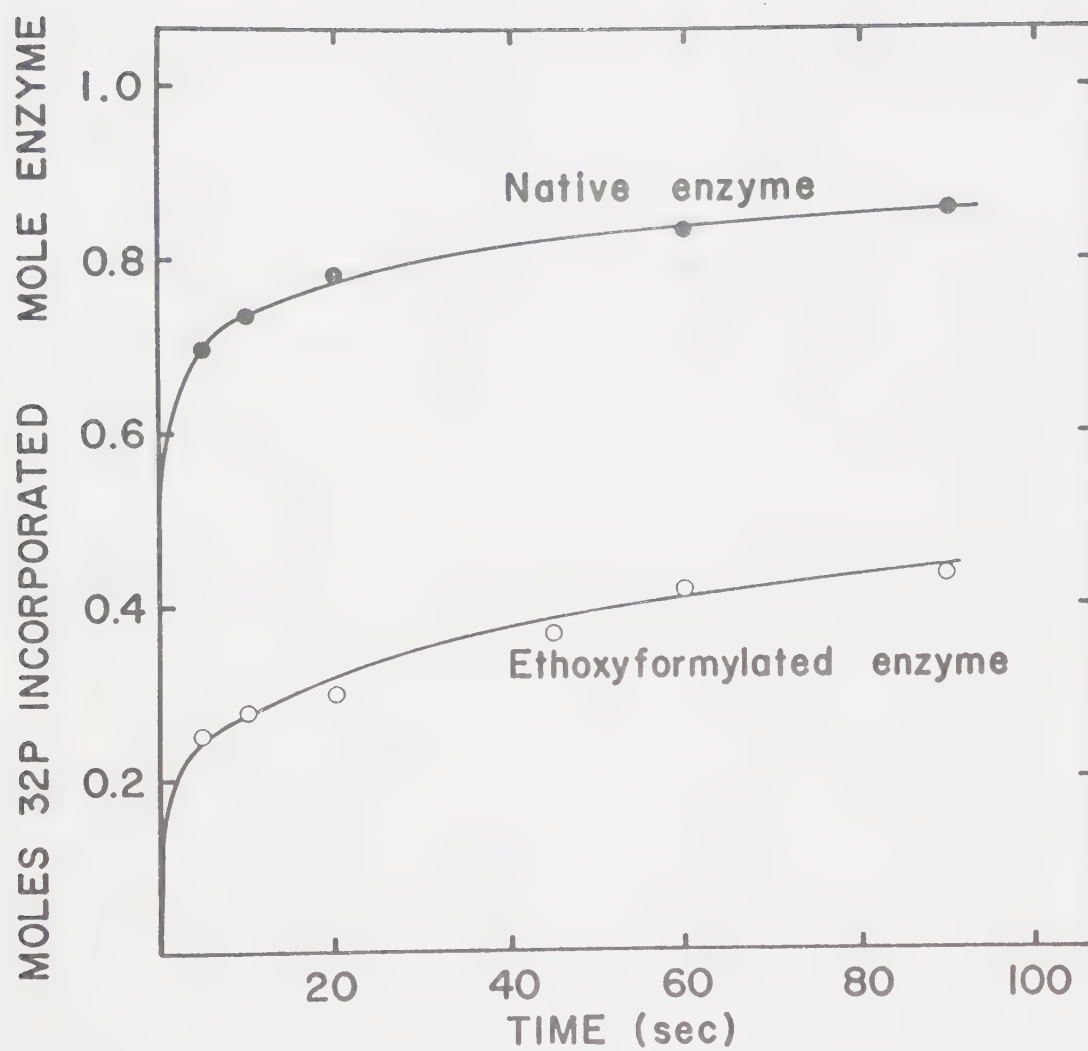
that it almost completely prevents the first half of the loss of activity. The failure of ATP or PEP to fully protect the activity could be due to modification of other histidine residues remote from the active site whose modification causes disruption of the enzyme structure. It has to be pointed out that the number of histidines modified in the presence of these substrates could not be determined spectrophotometrically due to high absorption of these substrates at 242 nm. However, the free phosphoenzyme was used to study the number of histidines modified. To our surprise, the rate of histidine modification of the phosphoenzyme was almost identical to that of the dephosphoenzyme under identical conditions.

Enzyme that was treated with the reagent until 6 residues of histidine were modified was tested for its ability to incorporate ^{32}P from ATP- β - ^{32}P . Fig. 31 shows that it was phosphorylated at about 50% of the rate of native enzyme. The modified enzyme incorporated 0.4 mole of phosphoryl group/mole enzyme/min whereas the native enzyme incorporated 0.8 mol/mole enzyme/min.

c. Reversibility of histidine modification by treatment with hydroxylamine

Fig. 30 also shows that if up to 8 - 10 histidine residues are modified, treatment with 0.3 N hydroxylamine will regenerate free histidyl residues and restore full enzyme activity. However, when 20 residues are modified, after hydroxylamine treatment only 10 residues are regenerated and only 25% of the activity is restored. The results indicate that only histidine residues are alkylated by the reagent unless the enzyme is extensively modified. Since hydroxylamine cleaves the ethoxyformyl-histidyl bond but does not cleave the more stable ethoxy-

Figure 31. Phosphorylation of ethoxyformylated enzyme. The enzyme (0.58 mg) was incubated with 0.3 mM ethoxyformic anhydride at pH 6.1 until the modification reached a maximum (6 histidine residues were modified per mole of enzyme). The treated enzyme was incubated with 0.78 mM ATP- β - ^{32}P + 5 mM MgCl_2 . The control is the untreated enzyme phosphorylated with ATP under the same conditions. Radioactivity incorporated into proteins was determined as described in the text.



formyl-lysyl bond (113), the inactivation of PEP synthetase by ethoxyformic anhydride appears to be primarily related to modification of histidyl residues only. The failure of hydroxylamine treatment to regenerate free histidines when more than 10 residues are modified could be due to conformational change as a result of further modification, which in turn buries some of the modified histidine residues to become inaccessible to NH_2OH . Alternatively, slow reaction with other side chains such as lysyl residues could account for the result.

d. Modification of thiol groups by ethoxyformic anhydride

Though above results indicate that the reagent modifies primarily histidyl residues, we have also checked the possibility of alkylation of other residues on the enzyme, both in the presence and absence of substrate, ATP. Grouselle et al. (101) reported that with the enzyme hexokinase the reagent also modified amino and thiol groups in the absence of substrate, whereas in the presence of glucose only amino groups were modified in addition to the histidyl residues.

When PEP synthetase was incubated with ethoxyformic anhydride, both in the presence and absence of ATP plus Mg^{2+} until 10 residues of histidine were modified, followed by hydroxylamine treatment, free histidine residues were regenerated. However, only 70% of its activity was restored for the enzyme treated in the presence of ATP whereas 100% was restored in the enzyme treated under the similar condition but in the absence of ATP. When the number of thiol groups on the enzyme was determined by DTNB titration, the enzyme treated in the absence of substrate contained 14 thiol residues as in the native enzyme. However, the enzyme treated in the presence of ATP showed only 10 SH- residues that were titratable with DTNB, indicating loss of 4 residues. This

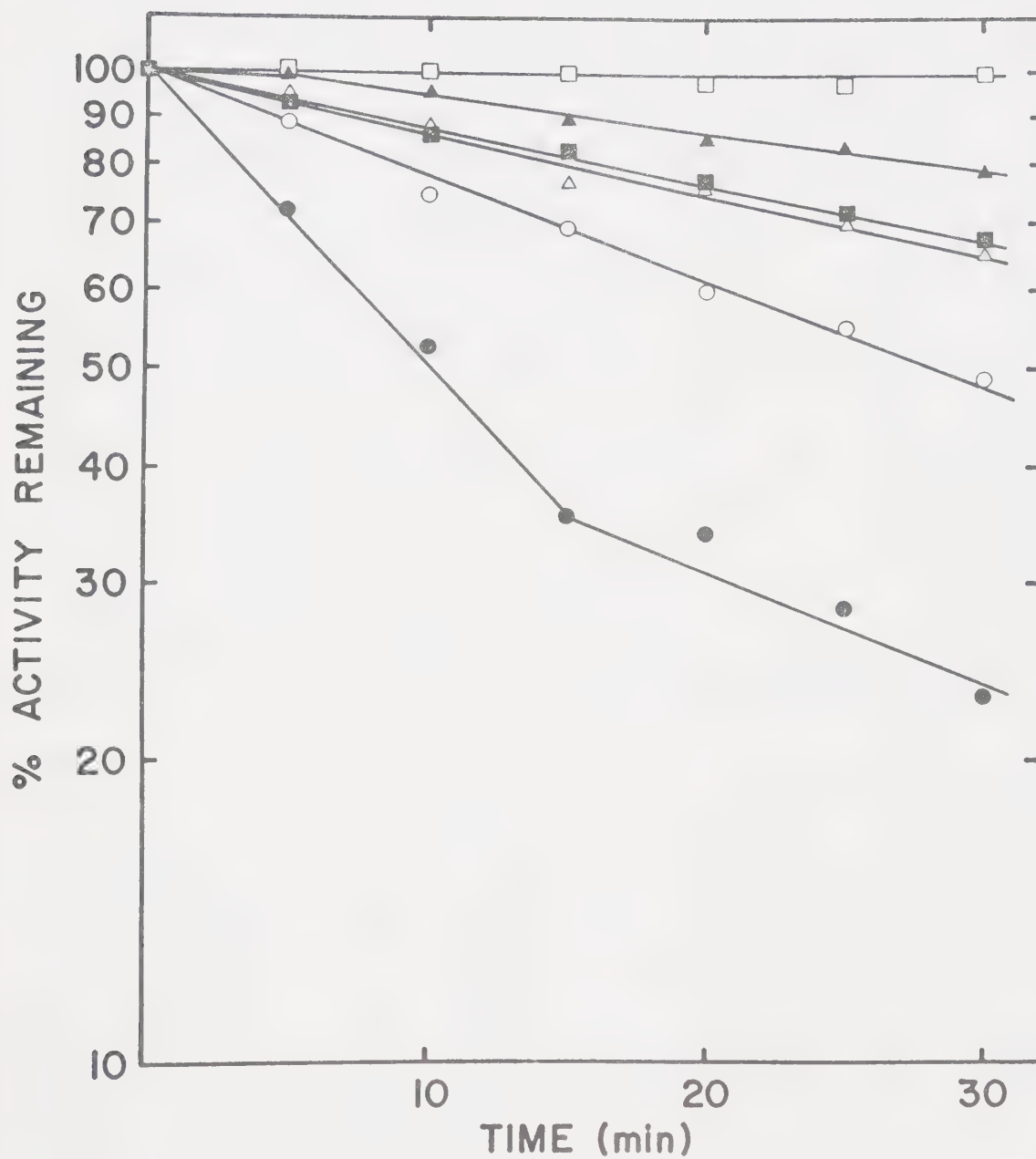
could be the result of structural rearrangement caused by ATP that makes some of the SH- groups become reactive toward ethoxyformic anhydride.

Although the effect of ATP on protection of enzyme activity against histidine modification is not as strong as expected, we remain confident that histidine is the site for phosphorylation as was indicated from analysis of the alkaline hydrolysate of the phosphoenzyme. Since the site of acylation by the reagent is unknown, i.e. whether it acylates on position 1 or 3 of the histidyl residues, it is possible that 3-phosphohistidine may still be susceptible to attack. In fact, this may depend on the nature of the environment surrounding particular residues. Some consideration must be given to the role of the particular histidyl residues in catalysis. It is conceivable that though the particular residue has been acylated on position 1, it can still be phosphorylated on position 3, though at a slower rate. This may explain the observed slower rate of phosphorylation of the modified enzyme. Moreover, the rate of hydrolysis of phosphoryl group from phosphohistidine or from N_3 -phosphoryl- N_1 -carbethoxylhistidine may not be very much different, since Benkovic and Sampson (118) reported that the rate of hydrolysis of N-phosphorylimidazole and that of N-phosphoryl-N'-methylimidazole are the same ($0.67 \times 10^{-4} \text{ min}^{-1}$).

3. Modification of arginyl residues and protective effect of substrates

When PEP synthetase was incubated with 10 mM phenylglyoxal, it was rapidly inactivated within 5 min. Fig. 32 shows the rate of enzyme inactivation by 2 mM phenylglyoxal and the effect of various substrates on the rate of loss of enzyme activity. All of the substrates protect the enzyme against inactivation by phenylglyoxal, although to different

Figure 32. Inactivation of PEP synthetase by phenylglyoxal. The enzyme was incubated with 2 mM phenylglyoxal in 50 mM MOPS pH 7.8 in the absence and presence of substrates. ●—●, control; O—O, plus 5 mM ATP + 5 mM MgCl_2 ; Δ — Δ , plus 5 mM pyruvate + 5 mM MgCl_2 ; ■—■, plus 5 mM AMP + 5 mM MgCl_2 ; ▲—▲, plus 5 mM PEP + 5 mM MgCl_2 ; □—□, plus 5 mM ATP + 0.1 mM oxalate + 5 mM MgCl_2 .



extents. The presence of ATP plus Mg^{2+} affords the least protection, and AMP plus Mg^{2+} or pyruvate plus Mg^{2+} protect the enzyme about equally. PEP is the most effective single substrate, but the enzyme activity is completely protected when ATP, Mg^{2+} and oxalate are present together.

From the study of phenylglyoxal inactivation, we infer that arginyl residues are a component of substrate binding site(s). That complete protection is provided by the presence of ATP and oxalate together suggests that an arginyl residue may be coordinated to the phosphoryl group undergoing transfer in the transition state. Since AMP alone is more effective than ATP, however, it must be borne in mind that when the enzyme is incubated with Mg^{2+} and ATP, this will result in formation of equivalent amounts of phosphoenzyme and AMP. Thus the effect of ATP could conceivably be due to the generation of AMP. Of course, it is possible that essential arginyl residues are located at sites on the protein that are distant from the ATP binding site but that in the presence of ATP they become inaccessible to phenylglyoxal because of induced conformational changes.

It was previously noted that arginyl residues are important in the binding of NAD to dehydrogenases, and it has been suggested that arginyl residues may serve the same function in enzymes that involve other phosphate-containing compounds such as NADP, FAD, coenzyme A, UDPG and ATP (106). It is clear that the structure of the guanidino group is remarkably well suited to interact strongly with a phosphate group (119). Recent work on alkaline phosphatase (107), aldolase (108), mitochondrial ATPase (120), aspartate transcarbamylase (121) and phosphorylase (122) indicates that arginyl residues function in binding of a variety of phosphorylated substrates. There is also evidence that

arginyl residues may play a role in the binding of substrate carboxyl groups (105, 123, 124). The present work is consistent with the suggestion that arginyl residues may participate in binding to the phosphoryl group of adenine nucleotide as well as to the carboxyl group of pyruvate or PEP. It is quite remarkable that arginine plays such a versatile role in the binding of cofactors and substrates to a wide range of enzymes.

CHAPTER VII

MODIFICATION OF PEP SYNTHETASE BY BROMOPYRUVATE; AN ACTIVE-SITE-DIRECTED REAGENT?

A. INTRODUCTION

Following the pioneering work of Meloche (125), bromopyruvate has been widely used in active-site studies as an alkylating analogue of pyruvate. It is a reactive alkylating reagent that is fairly specific for thiol groups at pH 4.6. It does not react at a significant rate with glycine, lysine, methionine, histidine or serine at pH 6.0 and 25° (126). Some of the more successful applications have been to identification of the cysteinyl residue at or near the active site of aspartate aminotransferase (127), 2-keto-3-deoxy-6-phosphogluconate aldolase (128), malic enzyme (129), isocitrate lyase (130), yeast alcohol dehydrogenase (131) and N-acetylneuraminic acid aldolase (126). In addition, bromopyruvate has been shown to esterify a carboxylate group in 2-keto-3-deoxy-6-phosphogluconate aldolase (128) and to alkylate a histidyl residue of carbonic anhydrase (132). This chapter reports the inactivation effect of bromopyruvate on PEP synthetase. Kinetic analyses of its inhibition indicate that bromopyruvate may act as a non-specific alkylating reagent of sulfhydryl residues of PEP synthetase, instead of the expected effect as an active-site directed reagent.

B. METHODS

1. Preparation of bromopyruvate solution

Bromopyruvate solution was prepared from bromopyruvic acid by adding NaOH until the pH was brought to pH 6.0. Its concentration was

determined by monitoring the change in absorbance at 340 nm in a system containing NADH and excess lactic dehydrogenase in 50 mM imidazole (pH 7.5). This method exploits the fact that, above pH 7.0, bromopyruvate is hydrolysed upon dilution to hydroxypyruvate, which is reduced by NADH in the presence of lactate dehydrogenase (125).

2. Inactivation studies

PEP synthetase inactivation studies were carried out at 25° in 50 mM phosphate buffer, pH 6.0. The enzyme was incubated with bromopyruvate at various concentrations, aliquots were taken at time intervals and the residual activity was determined by assays for both forward and reverse directions (46). The rate of inactivation by bromopyruvate was independent of assay method.

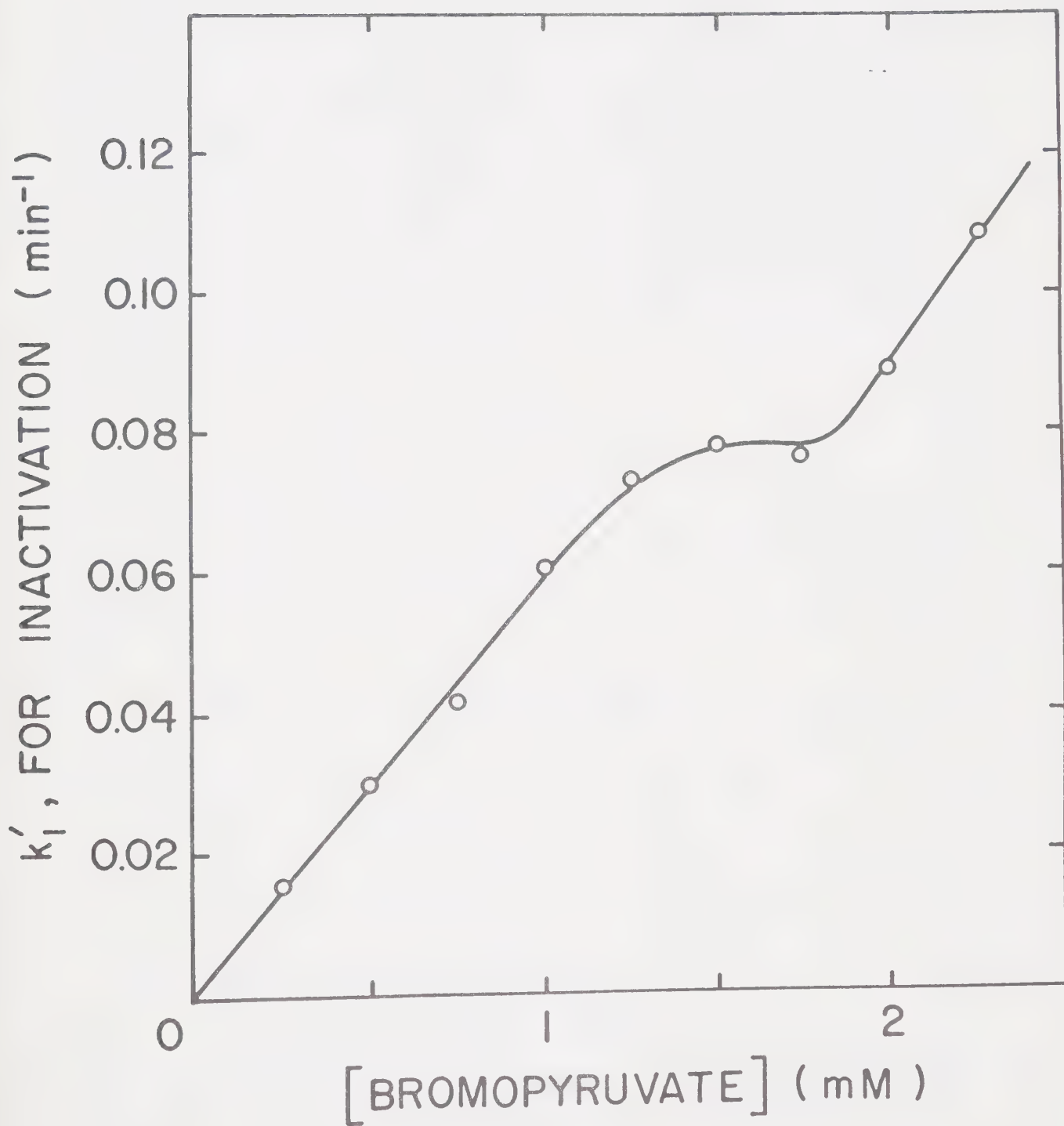
3. Determination of sulfhydryl residues in bromopyruvate-treated enzyme

The enzyme was incubated with 1 mM bromopyruvate, in the presence and absence of 4 mM pyruvate and 4 mM MgCl_2 , for 45 min. The treated enzyme was passed through a column of Sephadex G-50 to remove the excess reagents. It was then reacted with 0.5 mM DTNB and the number of residual sulfhydryl groups that reacted with DTNB was determined (112). Untreated enzyme was used as control.

C. RESULTS AND DISCUSSION

The time course of inactivation of PEP synthetase by bromopyruvate was studied. The plots of the logarithm of residual activity against time were linear at all concentrations of the reagent used, indicating that only one type of amino acid residue was modified. However, as shown in Fig. 33, the initial rate of inactivation is related to, but

Figure 33. Effect of bromopyruvate concentration on rate of enzyme inactivation. PEP synthetase was incubated with bromopyruvate (pH 6.0) at indicated concentration. Aliquots were taken at timed intervals to determine the residual activity. Rates of inactivation (k'_1) were determined from semi-log plots of residual activity versus time.



not proportional to, bromopyruvate concentration. At low concentration of bromopyruvate (up to 1.75 mM), the inactivation seems to obey saturation kinetics, whereas at concentrations above 2 mM, the rate of inactivation is very fast. At low concentrations, the effect of bromopyruvate may be due to its structural similarity with pyruvate, thus suggesting that bromopyruvate may alkylate within the active site of the enzyme.

The specificity of bromopyruvate for the enzyme's active site was tested by application of steady-state kinetics, as derived by Meloche (125). It is assumed that enzyme and bromopyruvate form a complex prior to inactivation.



where E represents free enzyme, I bromopyruvate, C_{inact} the enzyme-inactivator complex, and E_{inact} inactivated enzyme. It is further assumed that

$$[E_t] = [E] + [C_{\text{inact}}] + [E_{\text{inact}}] \quad (12)$$

where E_t represents the sum of all forms of the enzyme.

Finally, it is assumed that

$$v_{\text{inact}} = k_2 [C_{\text{inact}}] \quad (13)$$

$$\text{and } V_{\text{inact}} = k_2 ([E_t] - [E_{\text{inact}}]) \quad (14)$$

where v_{inact} represents the inactivation velocity at finite inactivator concentration [I] and V_{inact} is the inactivation velocity when [I] is infinite.

From the above assumption, the derived rate equation is

$$v_{\text{inact}} = \frac{V_{\text{inact}}}{\frac{K_{\text{inact}}}{[I]} + 1} \quad (15)$$

where K_{inact} is $(k_{-1} + k_2)/k_1$ and represents that concentration of bromopyruvate giving the half-maximum inactivation rate and effectively half-saturating the enzyme. The initial inactivation rates were determined from the time required for $[I]$ to cause a 50% inactivation (τ). Kinetically, $\tau = \ln 2 / \text{first order rate constant}$, thus $\tau \propto 1/v_{\text{inact}}$ and $T \propto 1/V_{\text{inact}}$.

Substituting these 2 terms in eq. 15, the linear form of the rate expression is

$$\tau = \frac{1}{[I]}(TK_{\text{inact}}) + T \quad (16)$$

The plot of τ vs. $1/[I]$ should give a straight line with intercept at T , the minimum inactivation half-time at infinite concentration of inactivator.

Fig. 34 shows that although a straight line is obtained from such a plot (using data obtained with bromopyruvate concentration below 2 mM), the fact that the minimum half-time of inactivation at infinite concentration of bromopyruvate (1 min) and its K_{inact} (9 mM) are much higher than expected from the data in Fig. 33 imposes some doubt that the reagent forms a dissociable complex with the enzyme prior to inactivation, as expected for an active-site direct reagent. Thus, bromopyruvate may act only as a non-specific alkylating reagent on the enzyme.

The ability of substrates to protect the enzyme against inactivation by bromopyruvate was also studied. Fig. 35 shows that the enzyme is protected by pyruvate but not by ATP. From studies of modification of

Figure 34. Inactivation half-time as a function of the reciprocal of bromopyruvate concentration. Data obtained from the results in Fig. 33.

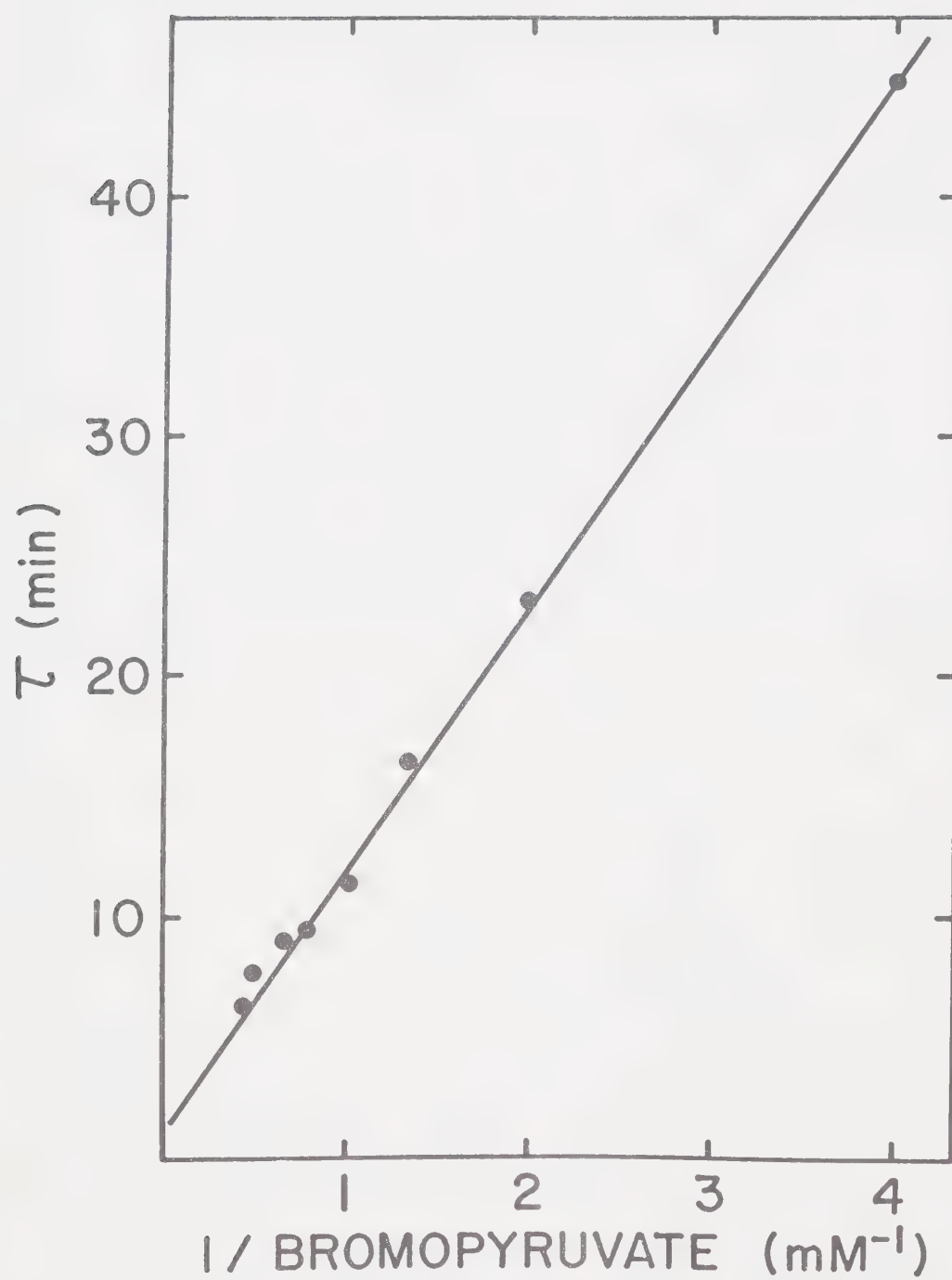
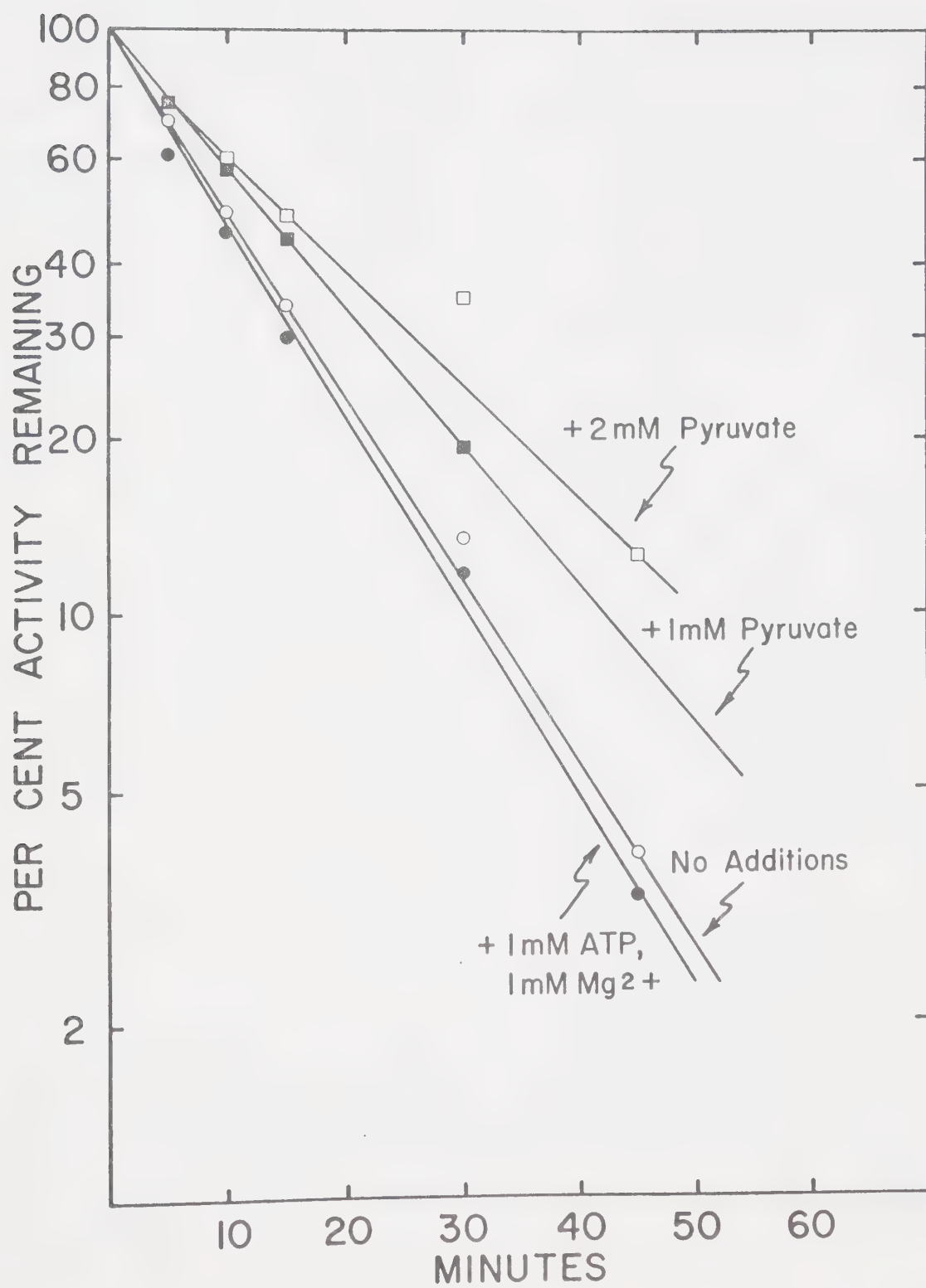


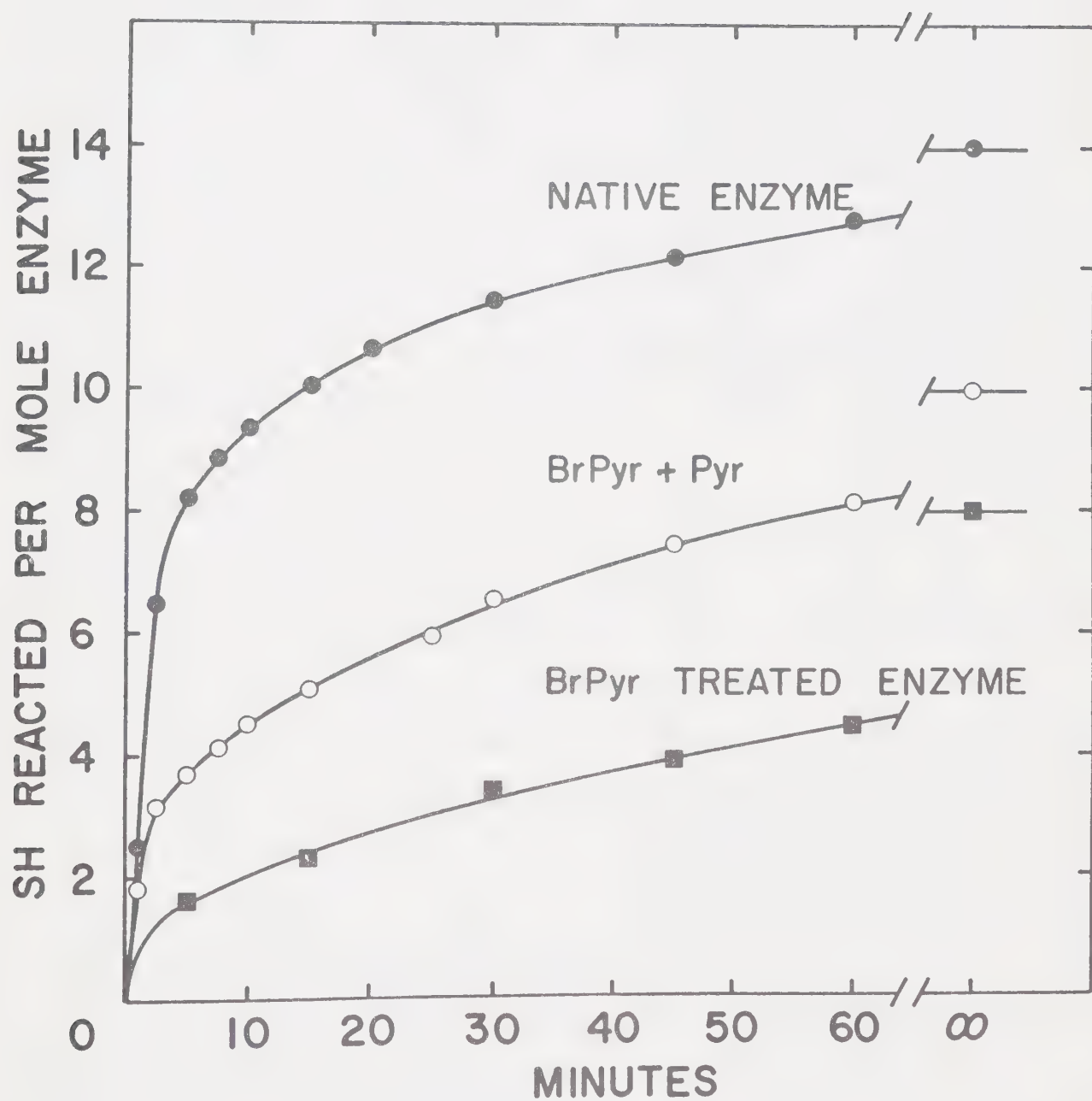
Figure 35. Effect of substrates on bromopyruvate inactivation. The enzyme was incubated with 1 mM bromopyruvate in the absence and presence of substrates as indicated.



sulfhydryl residues reported in Chapter VI, it was suggested that SH- residue is involved in the pyruvate binding site. Thus, inactivation by bromopyruvate could occur by alkylation at the same cystine residue. To test this, the enzyme was incubated with 1 mM bromopyruvate for 45 min. The treated enzyme was virtually inactive. It was then passed through a Sephadex G-50 column to remove all of the reagent and was subjected to SH- group determination by DTNB titration. Results shown in Fig. 36 indicate that under the conditions used, bromopyruvate alkylates 6 SH- groups which are fast-reacting in the native enzyme. However, when the enzyme was treated with the reagent in the presence of pyruvate, and the activity of the treated enzyme was 60% of that of native enzyme, 2 SH- residues/mole were protected as revealed by the increase in the number of residues titratable by DTNB (Fig. 36). From the results obtained, it seems that bromopyruvate does not only alkylate the pyruvate binding site but other SH- groups as well. Though pyruvate protects 2 SH- groups effectively, the fact that only 60% of enzyme activity is recovered suggests that those 4 SH- groups alkylated also play some important role either for catalysis or for maintaining the enzyme's conformation.

The rather non-specific behaviour of bromopyruvate on PEP synthetase is not surprising. It has been reported that bromopyruvate is also a powerful non-specific alkylating agent and it has been used as such (131, 133). Non-specific alkylation in addition to active-site - directed properties were also found with N-acetylneuraminic aldolase (126), aspartate aminotransferase (127) and the pyruvate dehydrogenase complex (134).

Figure 36. Titration of bromopyruvate-treated enzyme with DTNB. Details are given in the text.



CHAPTER VIII

NUCLEOTIDE SPECIFICITY OF PEP SYNTHETASE

A. INTRODUCTION

It appears that several phosphoryl group transferring enzymes show broad specificity with regard to the nucleotides which can act as substrates. Nucleoside diphosphokinase, for example, has degenerate nucleotide specificity, reacting with di- and triphosphate of ribonucleosides or deoxyribonucleosides that contain either purine or pyrimidine bases, in addition to some nucleotides that contain purine or pyrimidine analogues (137 - 139). Pyruvate kinase (140), creatine kinase (141), 3-phosphoglycerate kinase (142), phosphofructokinase (143) succinyl CoA synthetase (144) and adenylate kinase (145) whose true substrate is ATP have been found to be able to use dATP, GTP, ITP, TTP, CTP and UTP as substrates as well.

This chapter reports an investigation of the nucleotide specificity of PEP synthetase. Unlike the enzymes mentioned above, PEP synthetase cannot use GTP, ITP or CTP as substrates. However, it has been found that many ATP analogues modified on the sugar or purine ring moieties can be accommodated. This finding enables us to use the reverse reaction to synthesize a variety of ATP analogues without any need for the protection of reactive groups as was required for conventional chemical synthesis. From the variety of analogues tested we can infer which positions on ATP are important for substrate recognition.

B. METHOD

The assay conditions for the forward reaction were 0.1 M Tris-HCl,

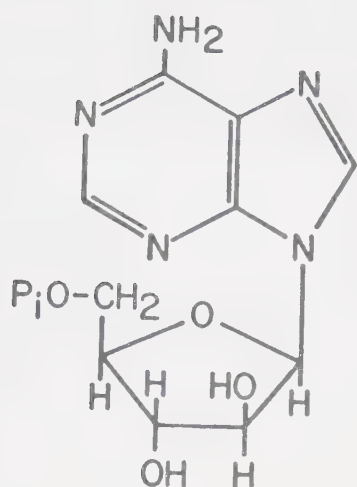
pH 8.0, 10 mM MgCl_2 , 2 mM pyruvate, 1 mM nucleoside-5'-triphosphate and 15 $\mu\text{g/ml}$ enzyme (specific activity 19 U/mg).

The reaction mixture for the reverse reaction was 0.1 M potassium phosphate pH 6.8, 20 mM MgCl_2 , 10 mM phosphoenolpyruvate, 10 mM nucleoside-5'-monophosphate and 130 $\mu\text{g/ml}$ enzyme. The reaction was incubated at 30°. Synthesis of NTP was monitored for up to 16 hours by thin layer chromatography on cellulose sheets with isobutyric acid/ammonium hydroxide/ H_2O (60/1/40 v/v) as solvent and on polyethyleneimine-cellulose sheets with 1.6 M LiCl as solvent (41). Reaction products were separated by DEAE-cellulose chromatography with elution achieved with a linear gradient from 0 - 0.6 M triethylammonium bicarbonate pH 7.5. Pooled fractions were concentrated by lyophilization.

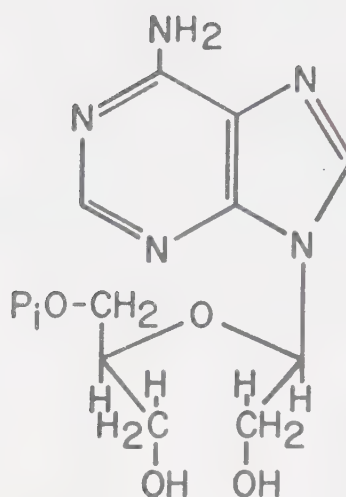
C. RESULTS AND DISCUSSION

Fig. 37 gives the structures of less common AMP analogues. The ability of various analogues to act as substrates of PEP synthetase for both the forward and reverse reactions is summarized in Table XI. As expected, when the reverse reaction was used to synthesize NTP, at no time was ribonucleoside-5'-diphosphate synthesis observed.

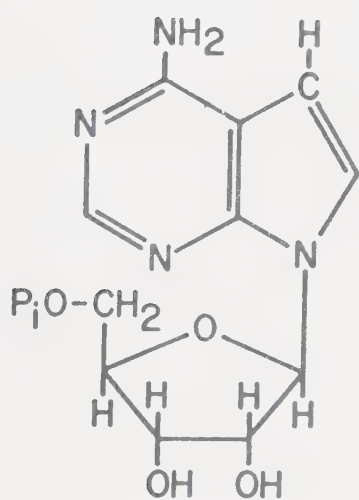
In the reverse direction, dAMP, araAMP, 3'-MeAMP, 6-MeAMP, TuMP, FMP and AMP can be phosphorylated with about 30% recovery of the NTP from the DEAE-cellulose column. 2'-MeAMP is converted to the triphosphate with about 15% recovery and 1-MeAMP is phosphorylated with 5 - 17% recovery. This variability is probably due to insolubility of this analogue under the reaction conditions. 6,6-Me₂AMP, AMP^{OX-RED} and α AMP (the 1' anomer of 5'-AMP) are not substrates and 6,2'-Me₂AMP is phosphorylated to a very low extent. The 6-keto nucleotides IMP and GMP,



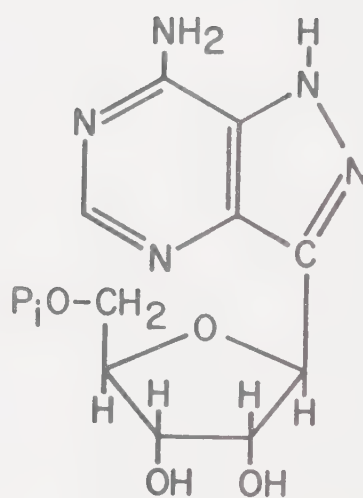
araAMP



AMP OX-RED



TuMP



FMP

Figure 37. Structure of some AMP analogues.

TABLE XI

Substrate Recognition by Phosphoenolpyruvate Synthetase

Compound	Reverse reaction: Isolation of NTP by DEAE cellulose chromatography ^{1,2}	Forward reaction: Initial reaction rate of analogue compared to ATP ¹
AMP/ATP	34	1.00
dAMP/dATP	33	0.33
2'-MeAMP	15	ND ⁴
3'-MeAMP	24	ND
1-MeAMP	5-17 ³	ND
6-MeAMP/6-MeATP	34	0.23
6,6-Me ₂ AMP	0	ND
6,2'-Me ₂ AMP	1	ND
araAMP/araATP	28	0.31
α -AMP	0	ND
AMP ^{OX-RED}	0	ND
TuMP/TuTP	29	0.71
FMP/FTP	28	0.42
GMP/GTP	0	0
IMP/ITP	0	0
CMP/CTP	0	0

¹As described in Methods.

²Percent of ultraviolet absorbing material ($\lambda = 260$ nm) eluting as NTP.

³Variable, see text.

⁴ND = not determined.

and the 4-amino pyrimidine nucleotide CMP cannot substitute for AMP in the reverse reaction, nor can the corresponding 5'-triphosphates substitute for ATP in the forward reaction.

Although dATP, 6-MeATP, araATP, TuTP and FTP are ultimately synthesized to the same extent as ATP, their relative initial rates for the forward reaction are very different. Compared to ATP these range from 0.23 for 6-MeATP to 0.71 for TuTP.

Several conclusions can be drawn from an evaluation of the substrate specificity of PEP synthetase. Firstly, modifications of the sugar moiety either on 2'- or 3'- positions appear unimportant for recognition by the enzyme, since AMP, dAMP, araAMP, 2'-MeAMP and 3'-MeAMP are all substrates. The lower yield of 2'-MeAMP may reflect a steric problem and this would seem to preclude the phosphorylation of AMP analogues containing bulky 2'- or 3'-substituents. Removal of the structural rigidity of the ribose ring as in AMP^{OX-RED} results in a loss of substrate recognition. As expected, the configuration at the anomeric center is also important since α AMP is not a substrate. Secondly, with regard to the heterocyclic base, variations in the imidazole ring are tolerated by the enzyme since both TuMP and FMP are substrates. Methylation of the 1-position does not result in a loss of recognition. If the 6-amino is monomethylated, recognition is not affected, but dimethylation at the 6-position results in complete loss of recognition and thus lack of synthesis. Replacement of the 6-amino by oxygen, as in IMP or GMP, results in no synthesis of the corresponding triphosphate. These last results indicate that of the various positions examined, only the 6-amino is clearly involved in substrate recognition, presumably by forming a hydrogen bond to the enzyme. Since the 4-amino pyrimidine

CMP is not phosphorylated, the purine ring moiety is necessary for enzyme recognition.

These results are in good agreement with previous reports about high specificity of PEP synthetase for nucleotide substrates which indicated that UTP, CTP or GTP could not replace ATP in the reaction with pyruvate (46, 146). A similar high degree of substrate specificity has been observed with pyruvate, phosphate dikinase from all sources. CTP, UTP, TTP, GTP, ITP and deoxy ATP have been tested as possible replacements for ATP without success, and TMP, UMP, CMP, GMP, IMP and ADP were unable to replace AMP (147, 168). For pyruvate, phosphate dikinase, when deoxy AMP was tried (148) it was found to be a poor substrate. However, deoxy ATP was not identified as the product of the reaction, and deoxy ATP could not replace ATP for the reaction with pyruvate. From the present contrasting studies with PEP synthetase, we have found that deoxy AMP may be freely substituted for AMP as a substrate for reverse reaction. The product, dATP, can be isolated and the test for its ability to replace ATP shows that it is about 33% as effective as ATP.

Other than gaining some insight about substrate recognition of the enzyme, this study also provides a useful method for the enzymatic synthesis of certain ATP analogues from their respective mononucleotides. In comparison to chemical methods of NTP synthesis (149), this enzymatic synthesis is limited both by substrate recognition and reaction scale since it is not suitable for the routine synthesis of mmols of NTP without a large enzyme supply. However, it is convenient for the synthesis of 25 - 50 μ moles of a particular NTP without the need for blocking agents and requires no "seed" NTP as does a previously described

enzymatic synthesis of FTP (150). Since the β -phosphate is derived from phosphoenolpyruvate and the γ -phosphate from buffer, specific radioactive labelling should also be possible.

CHAPTER IX

CONCLUSIONS

PEP synthetase has been purified to homogeneity. It appears to be a dimeric enzyme with a molecular weight of 150,000. The conclusion that it contains similar subunits is based upon the detection of only one protein band of MW 77,000 on SDS gel electrophoresis, and a comparison of the amino acid composition and the number of peptides detected in its tryptic fingerprints. Despite its dimeric structure, only one phosphoryl group has been found to be incorporated per enzyme molecule. This observation may be a consequence of negative cooperativity between the two active phosphorylation sites, or so-called "half-of-the-sites" reactivity (90). Furthermore, the saturation curves for phosphorylated substrates (ATP or PEP) which show an intermediary plateau region characteristic of negative cooperativity in various enzymes seem to support the above interpretation. Therefore, phosphorylation of the first site may cause some structural change that will shut off the phosphorylation of the second site. Various lines of evidence can be drawn upon to support the conclusion that there are conformational changes caused by the formation of phosphoenzyme. Firstly, from sedimentation equilibrium studies it is clear that the dephosphoenzyme exists as a loose dimer that tends to dissociate at low protein concentration, especially at room temperature, whereas the phosphorylation of the enzyme seems to tighten the dimeric structure of the enzyme both at 5° and 20°. Secondly, the circular dichroism study indicates some structural rearrangements caused by formation of the phosphoenzyme. Finally, indications of conformational change have been inferred from the effect of ATP on

the rate of DTNB titration. ATP protects some thiol residues but simultaneously accelerates the reaction of some other residues with the reagent. Furthermore, although ATP partially protects from inactivation by NEM, the NEM treated enzyme is able to be phosphorylated by ATP- β - ^{32}P . Thus the protection of thiol residues contributed by formation of phosphoenzyme could be due to a conformational change of the enzyme.

The correlation between phosphorylation of PEP synthetase and its conformational change may be proposed by a simple model that the dephosphorylated form of the enzyme exists as a loose dimer, and that the phosphorylation of either one of the active sites will promote some structural changes to make the enzyme become more compact. The resulting compact structure of the enzyme will make it difficult for the second active site to accommodate the second phosphoryl group, or if phosphorylation does occur it may be very unstable. A compact structure of enzyme caused by phosphorylation may also result in shielding of some and exposure of other reactive amino acid residues.

Phosphohistidine has been identified in the phosphorylated form of PEP synthetase. Three lines of evidence support this conclusion: (i) the pH-stability profile of the phosphoenzyme, (ii) the identification of 3- ^{32}P phosphohistidine in alkaline hydrolysates of ^{32}P -phosphoryl-PEP synthetase, and (iii) the comparison of the rate of acid hydrolysis of ^{32}P phosphoryl-PEP synthetase to that of succinyl CoA synthetase, an enzyme which is known to contain 3-phosphohistidine in its phosphorylated intermediate (78). Although cleavage by hydroxylamine is generally considered to be characteristic of an acylphosphate, it is known to occur with the phosphoramidate bond as well. Acetylphosphate, which may be considered a model compound for acylphosphoproteins,

has a k_2 of $42 \text{ min}^{-1} \text{ M}^{-1}$ for reaction with hydroxylamine (151) whereas the k_2 for phosphoramidate hydrolysis is $0.8 \text{ min}^{-1} \text{ M}^{-1}$ (152) under similar conditions. The k_2 for hydrolysis of phosphorylated PEP synthetase by hydroxylamine is $0.29 \text{ min}^{-1} \text{ M}^{-1}$, in the range expected for the phosphoramidate bond. In fact, other phosphoproteins containing phosphohistidine have been reported to be hydrolysed in the presence of hydroxylamine. Spronk et al. (27) have reported the identification of 3-phosphohistidine in the phosphorylated form of pyruvate, phosphate dikinase. Its phosphoenzyme has been reported to be hydrolysed in the presence of hydroxylamine with a k_2 of $0.50 \text{ min}^{-1} \text{ M}^{-1}$. Hays et al. (24) determined the k_2 of $0.55 \text{ min}^{-1} \text{ M}^{-1}$ for reaction of hydroxylamine with phosphorylated III^{lac} protein, which was also shown to contain 3-phosphohistidine. Thus, all of this is in accord with the phosphoryl group being bound to PEP synthetase via a phosphoramidate bond to a histidine residue. Theoretical considerations of the role of covalent intermediates in a catalysis suggest that the reactive amino acid on the enzyme must serve as a better attacking group than the final substrate and as a better leaving group than the leaving group on the initial substrate donor (153). The phosphohistidine intermediate of PEP synthetase may serve as such a catalytic group and may be considered high in chemical energy (32) and thus may meet these requirements. Furthermore, the flexibility of the imidazole side chain of histidine may help the transfer of the phosphoryl group to pyruvate as recently suggested by Milner and Wood (12) for catalysis by pyruvate, phosphate dikinase.

For enzymes at important branch-points of metabolism, allosteric regulation by metabolites has often been observed. For PEP synthetase, however, attempts to detect such effects by various metabolites have

failed to demonstrate any inhibition or activation of an allosteric nature. Product inhibition by AMP and the proper balance of AMP, ADP and ATP as defined as energy-charge seem to be responsible for the control system of the enzyme (14).

Like many enzymes using pyruvate as substrate, PEP synthetase is strongly inhibited by oxalate. This finding provides a useful tool to study the enzyme in its ternary complex in the chemical modification studies. As reported in Chapter VI, PEP synthetase is very sensitive to various sulfhydryl modifiers. However, the role of thiol residues in the activity of PEP synthetase has not been clarified. Although oxalate, a transition state analogue of pyruvate, protects the thiol residues from being modified by various reagents at all pH's studied, its incomplete protection leaves ambiguity about the role of thiol residues for pyruvate binding. Its involvement in ATP binding can be ruled out, however, by the fact that the NEM treated, inactive PEP synthetase can still be phosphorylated by ATP. Although ATP together with oxalate completely protects the enzyme from modification by NEM at pH 7.0, the inability of ATP to protect the enzyme from inactivation by iodoacetamide at pH 8.0 (the optimum pH for catalytic activity) makes it unlikely that the thiol residues are directly involved in the catalytic mechanism. It remains a possibility that a thiol residue may not be involved in direct interaction with pyruvate but is situated in the vicinity of the pyruvate binding site, and that its integrity is necessary for maintaining the active conformation of the enzyme. The presence of pyruvate might hinder reaction of such a residue with the sulfhydryl reagents.

Although results with modification of histidyl residues by ethoxyformic anhydride are not definitive, the fact that only ATP or PEP pro-

protects the enzyme at the early stage of modification gives us some further confidence that a histidyl residue is the site of phosphorylation of the enzyme.

The use of phenylglyoxal in modification studies has shown that, like other enzymes using nucleotides as substrate or cofactor, PEP synthetase has one or more arginyl residues which are essential for activity. The presence of both substrates, ATP and pyruvate, is necessary for complete protection of the enzyme from modification by phenylglyoxal. At present, we do not know whether the arginyl residues are essential for the catalytic mechanism or for substrate binding.

The high purity and substantial quantities of the enzyme we have at hand together with the information gathered from this investigation should encourage further study of PEP synthetase in terms of its structure and function relationship. The sequence of an active site peptide containing the phosphorylated residue could be obtained by application of the simple method developed by Wang *et al.* (77) which has been used successfully in isolation of peptide containing phosphohistidine from succinyl CoA synthetase (77), 3-phosphoglycerate mutase (80) and wheat germ acid phosphatase (154).

Furthermore, the possibility of double labelling of the enzyme at both the phosphorylated site and the pyruvate binding site is suggested by the fact that the inactive, alkylated enzyme can be phosphorylated. The results from sequencing may give some information as to whether these two sites are distant from each other in the primary structure. The clarification of the involvement of reactive amino acid residues in the catalytic mechanism or the conformation of the enzyme must await the application of additional techniques such as NMR and X-ray diffraction studies.

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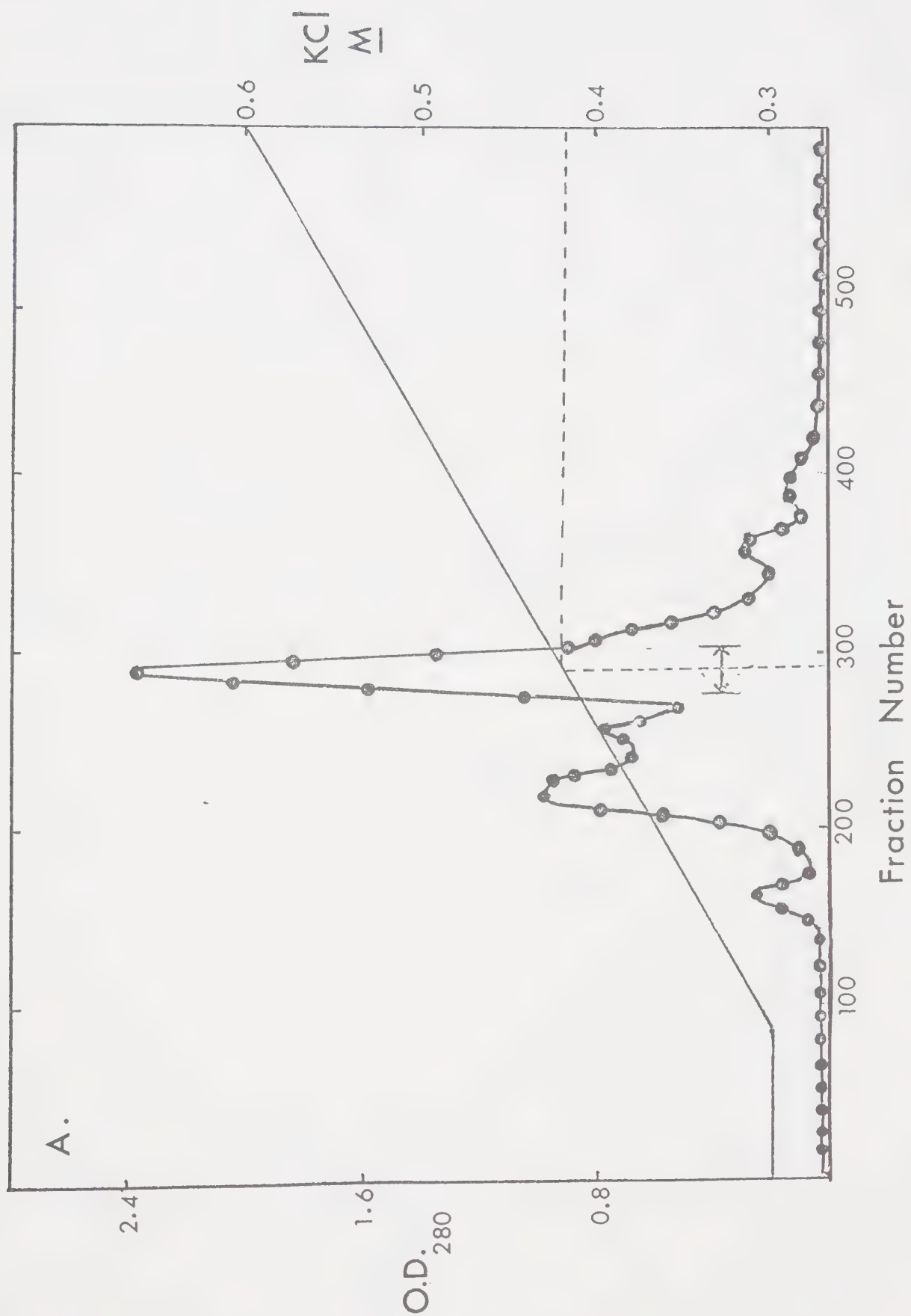
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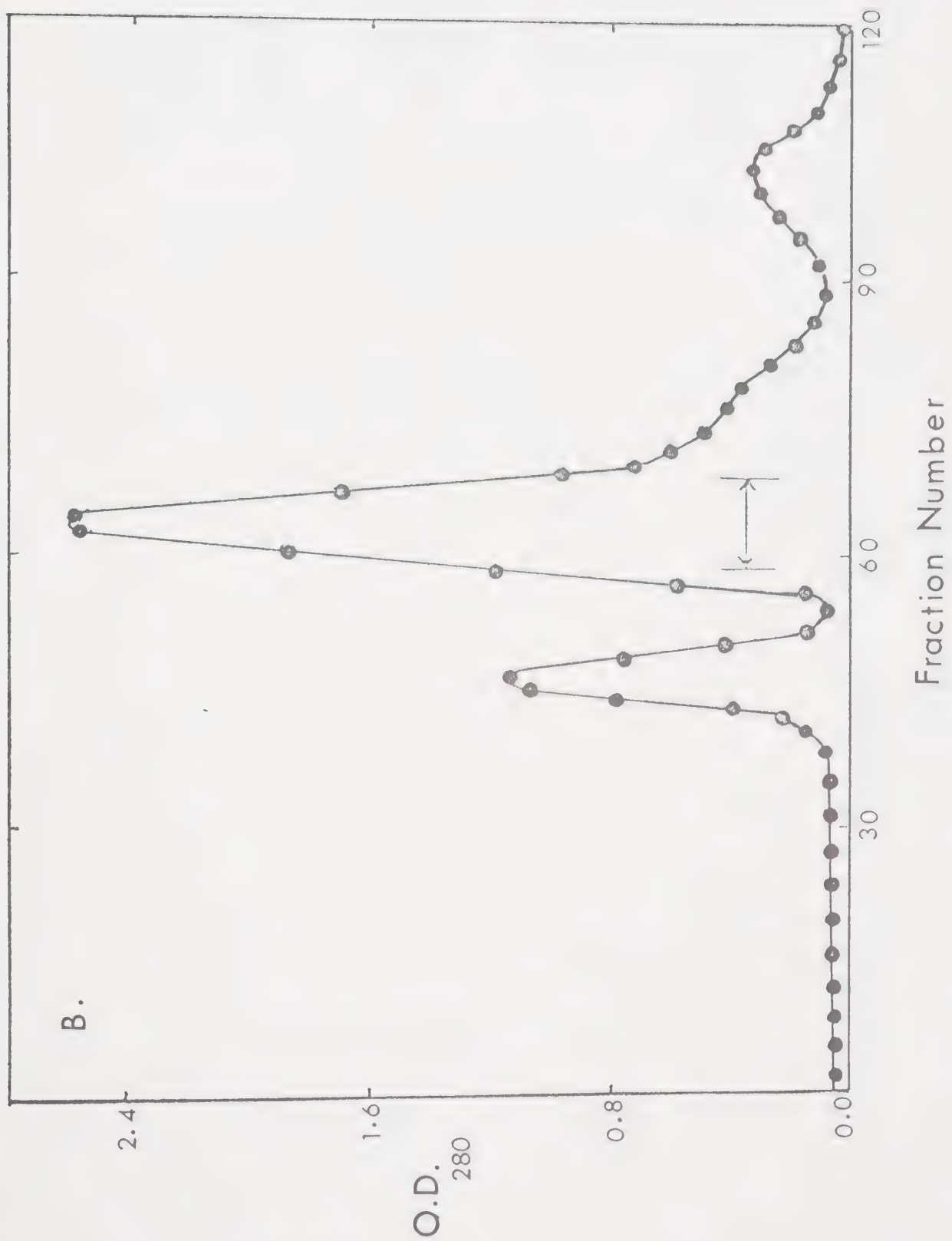
APPENDIX I

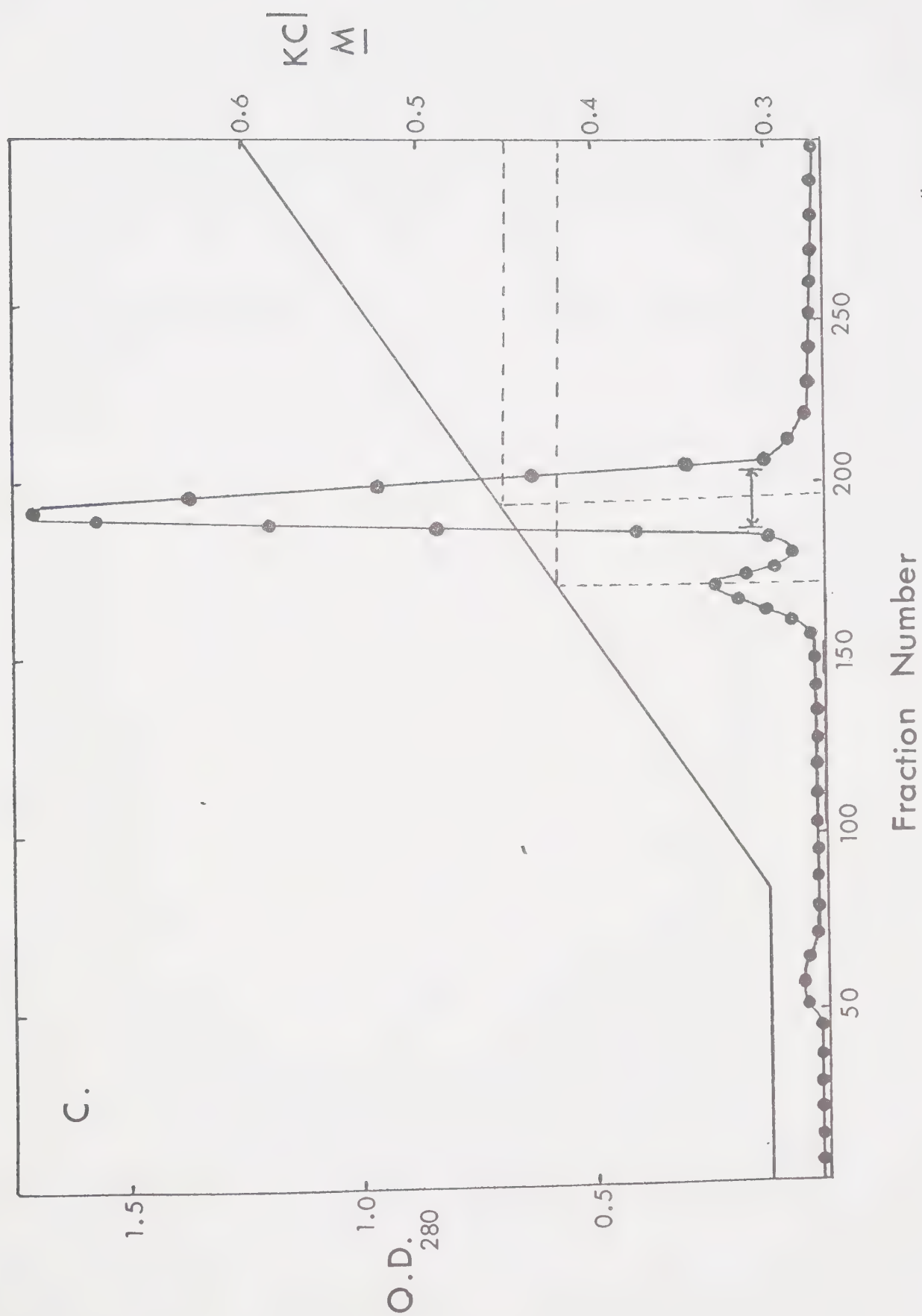
CHROMATOGRAMS OF ENZYME DURING THE PURIFICATION PROCEDURE

(Described in detail in Chapter III, Section B,1)

- A. Chromatogram of dephosphoenzyme on first QAE-A50 Sephadex column (6 x 50 cm). The enzyme was eluted with linear gradient of 0.3 M KCl to 0.6 M KCl in 0.03 M MOPS - 2 mM EDTA - 2 mM mercaptoethanol, pH 6.8 (total volume = 10 litres). The enzyme was eluted at a salt concentration of 0.42 M KCl.
- B. Chromatogram of the pooled fractions of enzyme obtained from (A) on a Sephadex G-200 column (3 x 100 cm). The column was equilibrated and eluted with 50 mM Tris, 0.2 mM EDTA and 0.2 mM DTT, pH 6.8.
- C. Chromatogram of phosphoenzyme on QAE-A50 Sephadex column. The pooled fractions of enzyme obtained from the Sephadex G-200 step were treated with 1 mM ATP + 1 mM MgCl_2 in 50 mM Tris-HCl pH 8.0. The small molecular weight components were removed by means of gel filtration on a Sephadex G-50 column. The phosphoenzyme was re-chromatographed on the second column of QAE-A50 Sephadex (3 x 50 cm) and was eluted with a linear gradient of 0.3 M KCl to 0.6 M KCl (total volume = 4 litres). The phosphoenzyme was eluted at a salt concentration of 0.45 M KCl.







APPENDIX II

PUBLICATIONS ARISING FROM THIS WORK

1. Doug Johnson, Malcolm MacCoss and Suree Narindrasorasak (1976)
Biochem. Biophys. Res. Comm. 71, 144.
2. S. Narindrasorasak and W.A. Bridger (1977) J. Biol. Chem. (in
press).

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